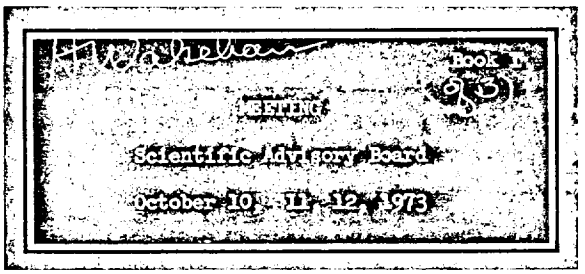

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9. MISCELLANEOUS

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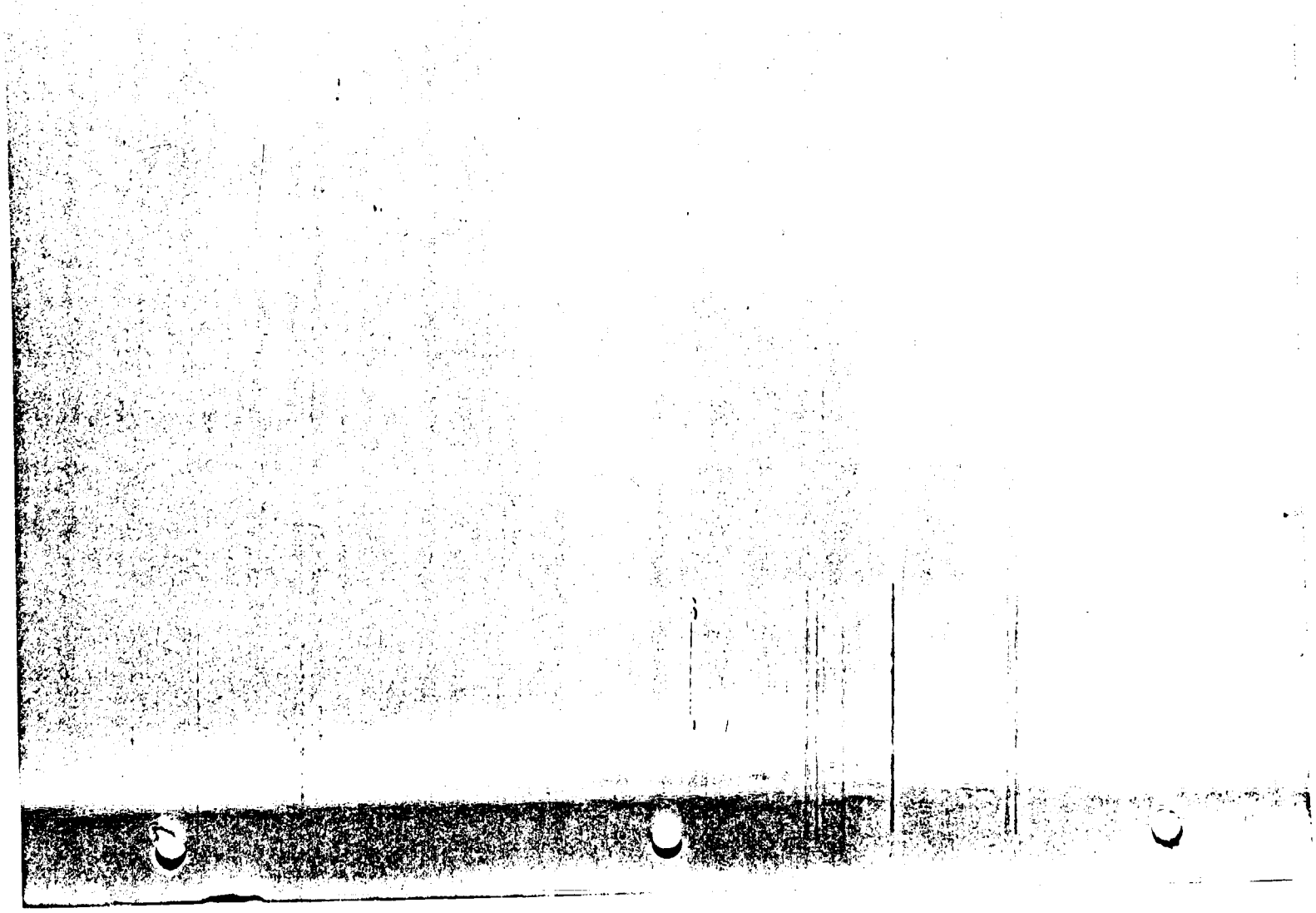
AGENDA AND MINUTES



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AGENDA

SCIENTIFIC ADVISORY BOARD

October 10, 11, 12, 1973

1. Interim actions to be considered or confirmed:

   a. Administrative

   b. Executive Committee, SAB

2. Applications for Grants:

   Book I - Cancer

   Book II - Pulmonary

   Book III - Cardiovascular
   Pharmacology
   Miscellaneous

3. Other business

4. Future meeting dates

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The Council For Tobacco Research–U.S.A., Inc.

Minutes Approved

CONFIDENTIAL REPORT

SCIENTIFIC ADVISORY BOARD MEETING

NEW YORK, NEW YORK

MARCH 14-15-16, 1973

Dr. Sheldon C. Sommers, Chairman	
Dr. Howard B. Andervont	
Dr. Richard J. Bing	
Dr. McKeen Cattell	
Dr. William U. Gardner	Scientific Consultant, CTR
Dr. Robert J. Huebner	
Dr. Leon O. Jacobson	
Dr. Clayton G. Loosli	
Dr. Hans Meier	
Dr. John P. Wyatt	
Dr. Murray Senkus	Chairman, ITC
Dr. Helmut R. R. Wakeham	Chairman Elect, ITC
Mr. H. H. Ramm	President, CTR
Mr. W. T. Hoyt	Executive Vice President, CTR
Dr. Robert C. Hockett	Acting Scientific Director, CTR
Dr. J. Morrison Brady	Associate Scientific Director, CTR
Dr. John H. Kreisher	Associate Scientific Director, CTR
Dr. Frederic W. Nordsiek	Associate Scientific Director, CTR
Dr. Vincent F. Lisanti	Scientific Associate, CTR
Dr. Arthur Furst	Research Consultant, CTR
Mr. Leonard S. Zahn	Consultant, CTR

1. The report of the September 1972 meeting of the Scientific Advisory Board was approved.

2. The report, actions and recommendations of the December 8, 1972 meeting of the Planning Committee were approved, including the following:

   $100,000.00 for reference cigarettes

   $ 62,500.00 to cover cost of rebuilding Walton Horizontal Smoke Exposure machines

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3. The following administrative actions were approved:

467-B Thomas C. Westfall, Ph.D. Permission was granted to extend this grant from December 31, 1972 to March 31, 1973 without additional funds.

519-BS Louis A. Soloff, M.D. A supplementary grant was approved in the amount of $5,000.00 to provide additional funds for purchase of consumable supplies and certain permanent equipment vital to the current project.

547-C Joseph J. Guarneri, Ph.D. Permission was granted to carry forward the unexpended balance of $549.59 to 547-CR1.

548-A S. N. Pradhan, M.D., Ph.D. Permission was granted to extend this grant for a second time from December 31, 1972 to September 30, 1973 with no additional funds.

623-BR1 Walter B. Essman, Ph.D. Permission was granted (1) to use unexpended funds in the approximate amount of $4,190.77, and (2) to extend the project from January 31, 1973 to March 31, 1973.

646-AR1 Robert C. Rosan, M.D. Permission was granted to transfer $4,900.00 from Salaries to Equipment for the purchase of an electrophoresis scanner.

646-A An uncommitted balance of $3,256.97 was returned and deposited.

702R2 Albert H. Niden, M.D. An unexpended balance of $204.51 was received and deposited.

704 Lucile Smith, Ph.D. An unexpended balance of $1,321.50 was received and deposited.

734R2 Carroll E. Cross, M.D. Permission was granted to transfer $1,200.00 from Salaries to Supplies, and $1,800.00 from Salaries to Equipment. Since overhead is not allowed for Equipment, a check for $270.00 (representing 15% of $1,800.00) was returned by the University authorities and deposited. The grant, therefore, has been reduced from $23,175.00 to $22,905.00.

764R1 Charles G. Cochrane, M.D. Permission was granted to carry forward the unexpended balance of $644.30, thus reducing 764R2 from $31,269.00 to $30,624.70.

808 Allen B. Cohen, M.D. An unexpended balance of $327.50 was returned and deposited.

814 Una Smith, Ph.D. The September minutes reported a time extension without additional funds from September 30, 1972 to January 31, 1973. This has been amended from September 30 to December 31, 1972. The renewal grant (814R1) therefore became effective on January 1, 1973.

826 Thomas R. Dawber, M.D. Permission was granted to carry forward the unexpended balance of $11,324.64 to the current grant 826R1.

833 A. Stanley Weltman, Ph.D. An unexpended balance of $3,665.71 was received and deposited.

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839 Edwin R. Fisher, M.D. Permission was granted to carry forward the unexpended balance of $6,246.00 to the current grant 839R1.

844 Duane G. Wenzel, Ph.D. Permission was granted to extend this grant from June 30, 1973 to June 30, 1974 without additional funds.

854 Miss Eleanor J. Macdonald. Permission was granted to carry forward the unexpended balance of $794.31 for the purposes of the project.

864 T. A. Slotkin, Ph.D. The September minutes reported the amount approved as $12,109.00. The correct amount approved was $12,110.00.

869 Ronald P. Rubin, Ph.D. Permission was granted to reallocate $2,425.00 from Equipment to Salaries.

891 Dr. Georg B. Neurath. The December 1972 Planning Committee meeting minutes reported the amount approved as $41,007.50. The correct amount approved was $41,000.00.

Contract 1-C Mason Research Institute. The December 1971 minutes approved an appropriation in the amount of $7,000.00 and in the March 1972 minutes $15,000.00 to defray the salary and expenses of Dr. Charles W. Nystrom of R. J. Reynolds Tobacco Company. These totaled $13,437.19, and the balance remaining of $8,562.81 is cancelled.

2-D Microbiological Associates, Inc. An extension from February 1 to March 31, 1973 was approved on a pro rata basis not to exceed a total of $25,000.00 during that period.

3-B IIT Research Institute. The following per diem and expenses were paid to Dr. G. P. Brierly for consultation and work on the contract: Per Diem $240.00 and Expenses $165.00, totaling $405.00.

This contract was cancelled as of March 31, 1973.

6-B IIT Research Institute. This contract was cancelled as of March 31, 1973.

9-B University of San Francisco. Expenses and per diem in the amount of $442.50 for the period July 1 to September 30, 1972 were approved for Dr. Robert M. Kovatch, and $624.50 for the period October 1 to December 31, 1972.

Expenses and per diem in the amount of $417.15 for Dr. Jay A. Levy were approved.

4. The following changes in operating procedure as described by Dr. Sommers and Mr. Ramm were approved:

(1) An Executive Committee of the Advisory Board will replace the Planning Committee which had been formed as a stopgap operation when Dr. Little became less active. Since the Board now meets twice a year, the Executive Committee will convene as needed during the intervals between meetings, and planning work will be a function of the scientific staff

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of The Council. The Executive Committee was confirmed composed of Dr. Sommers, Chairman, Dr. Jacobson, Dr. Bing, Dr. Loosli, Dr. Wyatt and Dr. Gardner, ex officio. Any three members constitute a quorum and are authorized to take actions on behalf of the Board as needed.

(2) The 1973 budget is earmarked to a large extent for specific projects which are planned, rather than unsolicited ones. In order to pursue planned projects and to cull out and integrate desirable application into The Council's program from the increasing number of unsolicited applications, all projects in future will be evaluated by the Scientific Advisory Board or, in its stead, by the Executive Committee. After such evaluation, the Scientific Director, or the Acting Scientific Director, will decide which projects shall be funded and at what rate.

5. The following renewal applications were recommended for approval, subject to the determination by the Acting Scientific Director as to priority and the amount to be funded:

24R17	Paul S. Larson, Ph.D.	49,795.00	
310R12	Richard J. Bing, M.D.	38,893.00	
519-BR1	Louis A. Soloff, M.D.	85,000.00	
547-CR2	Joseph J. Guarneri, Ph.D.	22,944.00	
573-BR2	Clayton G. Loosli, M.D., Ph.D.	181,944.00 $84,000.00 was recommended for nine additional months. This figure was based on a pro rata of $112,000.00 per year.	
623-BR2	Walter B. Essman, M.D., Ph.D.	21,579.00	Terminal
758-BR1	Hans Meier, D.V.M.	23,310.00	
763-AR2	John W. Parker, M.D.	37,950.00	Terminal
786R2	H. Hugh Fudenberg, M.D.	61,737.00	Terminal
802R2	Attallah Kappas, M.D.	49,925.00	Terminal
808R2	Allen B. Cohen, M.D., Ph.D.	12,487.00	Terminal
826R2	Thomas R. Dawber, M.D.	54,625.00	Terminal
838R1	Baruj Benacerraf, M.D.	62,110.00	
844R1	Duane G. Wenzel, Ph.D.	12,264.00	Terminal
847R1	Wayne L. Ryan, Ph.D.	55,261.00	Terminal
857R1	Jerome I. Kleinerman, M.D.	16,414.00	Terminal
862R1	James Travis, Ph.D.	22,237.00	

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6. The following renewal application was rated for disapproval:

486-BMR1	Anthony A. Albanese, Ph.D.	16,606.00

7. The following contract proposals were recommended for approval subject to the determination by the Acting Scientific Director as to priority and the amount to be funded:

Contract	9-C	University of San Francisco	139,139.00
	I4	Microbiological Associates Incorporated	350,000.00
	15	Oak Ridge National Laboratory	208,500.00

8. The following new applications were recommended for approval subject to the determination by the Acting Scientific Director as to priority and the amount to be funded:

455-B	Benjamin Bell, M.D.	64,000.00
588-D	B. Bhagat, Ph.D.	36,805.00
599-C	Domingo M. Aviado, M.D.	39,984.00
629-D	Carlton K. Erickson, Ph.D.	16,735.00
642-B	Leonide Goldstein, D.Sc.	28,579.00
725-CM	Jack Chalon, M.D.	18,110.00
741-A	Joseph M. Lauweryns, M.D., Ph.D.	38,806.00
764-B	Charles G. Cochrane, M.D.	37,892.00
766-A	Richard A. Lerner, M.D.	64,385.00
787-A	Gary D. Friedman, M.D.	101,100.00
870	H. J. Eysenck, Ph.D., Sc.D.	21,562.00
879	H. David Mosier Jr., M.D.	29,513.00
883	Ronald R. Hutchinson, Ph.D.	14,030.00
897	Robert C. Rosan, M.D.	54,615.00
898	Norman W. Heimstra, Ph.D.	12,592.00
899	Martha Greenwood, M.D.	24,035.00

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9. The following new applications were deferred pending receipt of additional information:

888	T. J. Yang, Ph.D.	43,692.00
901	George Weinbaum, Ph.D.	45,332.00

10. The following new applications were rated for disapproval:

776-A	Elliot S. Vesell, M.D.	34,730.00
880	Kurt Amplatz, M.D.	51,449.00
881	Victor Milstein, Ph.D.	6,843.00
882	R. Robinson Baker, M.D.	69,411.00
884	Albert Castro, Ph.D.	48,545.00
886	Harold J. Sobel, M.D.	31,050.00
887	Alvin H. Sacks, M.D.	42,197.00
890	Frederick L. Schaffer, Ph.D.	32,727.00
893	F. W. Grant, Ph.D.	29,125.00
894	Luigi Valzelli, M.D.	21,748.00
896	Joel Warren, Ph.D.	22,994.00
900	Victor J. Schenker, Ph.D.	35,410.00
902	Morton Galdston, M.D.	43,492.00
903	Joseph Mendicino, Ph.D.	31,230.00
Case 164	Paola S. Timiras, M.D., Ph.D.	

11. It was recommended that the following contracts be closed out as soon as practicable:

Contract	4	Bio-Research Institute, Incorporated
	5	Bio-Research Consultants, Inc.

12. The next meeting of the Scientific Advisory Board was scheduled to be held on October 10-11-12, 1973.

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13. The Chairman expressed the gratitude of the Board to Dr. Murray Senkus for his efforts and cooperation with the Board during his term as Chairman of the Industry Technical Committee.

The Chairman welcomed Dr. Helmut R. R. Wakeham as the incoming Chairman of the Industry Technical Committee for a term of two years.

14. Subsequent to the meeting, of the renewal applications recommended for approval and referred to in paragraph 5, the Acting Scientific Director determined that the following should be funded in the respective amounts indicated:

24R17	Paul S. Larson, Ph.D.	49,795.00	
310R12	Richard J. Bing, M.D.	38,893.00	
519-BR1	Louis A. Soloff, M.D.	62,500.00	
547-CR2	Joseph J. Guarneri, Ph.D.	22,944.00	No assurance of further support.
573-BR2	Clayton G. Loosli, M.D., Ph.D.	84,000.00	for nine months, Oct. 1, '73 to June 30, '74.
623-BR2	Walter B. Essman, M.D., Ph.D.	21,579.00	Terminal
758-BR1	Hans Meier, D.V.M.	23,310.00	
786R2	H. Hugh Fudenberg, M.D.	61,737.00	No assurance of further support.
808R2	Allen B. Cohen, M.D., Ph.D.	12,487.00	No assurance of further support.
826R2	Thomas R. Dawber, M.D.	54,625.00	No assurance of further support.
838R1	Baruj Benacerraf, M.D.	50,000.00	No assurance of further support.
857R1	Jerome I. Kleinerman, M.D.	16,414.00	Terminal
862R1	James Travis, Ph.D.	22,237.00	

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15. Subsequent to the meeting, of the contract proposals recommended for approval and referred to in paragraph 7 above, and those recommended to be closed out in paragraph 11 above, the Acting Scientific Director determined that the following should be funded in the respective amounts indicated:

Contract	4	Bio-Research Institute, Incorporated	31,800.00
	5	Bio-Research Consultants, Inc.	26,872.00
	9-C	University of San Francisco	114,856.00
	14	Microbiological Associates Incorporated	350,000.00
	15	Oak Ridge National Laboratory	208,500.00

16. Subsequent to the meeting, of the new applications recommended for approval and referred to in paragraph 8 above, the Acting Scientific Director determined that the following should be funded in the respective amounts indicated:

455-B	Benjamin Bell, M.D.	64,000.00	
599-C	Domingo M. Aviado, M.D.	25,000.00	
642-B	Leonide Goldstein, D.Sc.	28,580.00	No assurance of further support. $14,290.00 of this amount had previously been committed at the Dec. 8, '72 meeting of the Planning Committee in paragraph 2 above.
725-CM	Jack Chalon, M.D.	18,110.00	No assurance of further support.
741-A	Joseph M. Lauweryns, M.D., Ph.D.	21,090.00	No assurance of further support. $10,546.00 of this amount had previously been committed at the Dec. 8, '72 meeting of the Planning Committee in paragraph 2 above.
764-B	Charles G. Cochrane, M.D.	11,000.00	Terminal
766-A	Richard A. Lerner, M.D.	47,385.00	1003543817
787-A	Gary D. Friedman, M.D.	101,100.00	$25,275.00 of this amount had previously been committed at the Dec. 8, '72 meeting of the Planning Committee in paragraph 2 above.
870	H. J. Eysenck, Ph.D., Sc.D.	21,000.00	in U.S. dollars.
897	Robert C. Rosan, M.D.	2,175.00	Terminal.
898	Norman W. Heimstra	12,592.00	

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

17. Subsequent to the meeting, the Acting Scientific Director determined that the recommendations for approval by the Planning Committee, referred to in paragraph 2, should be funded in the respective amounts indicated:

$100,000.00 for reference cigarettes.

$ 62,500.00 to cover cost of rebuilding Walton Horizontal Smoke Exposure Machines.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

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INTERIM ACTIONS

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

ADMINISTRATIVE REPORT

381R2 Barbara B. Brown, Ph.D. A balance of $2,248.75 remained after completion of this project and is therefore cancelled.

550-AMR2 John E. Craighead, M.D. An unexpended balance of $584.62 was received and deposited.

603-AR2 Shirley L. Kauffman, M.D. Permission was granted to carry forward the unexpended balance of $3,835.44 to grant 603-B.

623-BR1 Walter B. Essman, Ph.D. A balance remained of $450.00 after completion of this project and is therefore cancelled.

688-A Sheldon C. Sommers, M.D. An unexpended balance of $11,202.98 was received and deposited.

702 Albert H. Niden, M.D. Through an administrative error the final quarter of this grant was not paid and, therefore, a check in the amount of $4,253.75 was sent to Temple University to close out this project.

702-A A check in the amount of $4,000.00 was received from Temple University representing the estimated refund after Dr. Niden's transfer to Drew Postgraduate Medical School effective June 1, 1973. This money was deposited and a check drawn to Drew to aid Dr. Niden in starting up the project in the interim.

763-A John W. Parker, M.D. Permission was granted to carry forward the unexpended balance of $117.24 to 763-AR1.

763-AR1 Permission was granted to transfer $800.00 from Supplies to Salaries.

Permission was granted to extend this project from July 1 to December 31, 1973.

787-A Gary D. Friedman, M.D. An unexpended balance of $5,182.65 remained from 787R1 and this was deducted from the present grant authorization of $101,100.00, making the final authorization $95,917.35.

808R1 Allen B. Cohen, M.D. An unexpended balance of $2,305.94 was returned and deposited.

814R1 Una Smith, Ph.D. Permission was granted to transfer $400.00 from Supplies to Travel.

864 Theodore Slotkin, Ph.D. Permission was granted to transfer $3,025 from Supplies to Equipment.

Contract 3-B IIT Research Institute. After having been cancelled as of March 31, 1973 and funding requirements settled as of that date, a balance of $12,398.32 remained which is cancelled.

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Contract 6-B IIT Research Institute. After having been cancelled as of March 31, 1973 and funding requirements settled as of that date, a balance of $16,168.44 remained which is cancelled.

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

The following authorizations were approved at a staff meeting on August 2, 1973:

709-CS Lucio Severi, M.D. This project was extended for the months of September and October 1973 in a pro rata amount of $2,500.00 so that the grantee can continue his project until a decision as to his application is made at the SAB meeting in October.

836-AS Walter B. Essman, Ph.D., M.D. This project was extended for the month of October 1973 in a pro rata amount of $3,334.00 so that the grantee can continue his project until a decision as to his application is made at the SAB meeting in October.

839R1S Edwin R. Fisher, M.D. This project was extended for the month of October 1973 in a pro rata amount of $2,108.00 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

868S Herbert McKennis Jr., Ph.D. This project was extended for the month of October 1973 in a pro rata amount of $5,000.00 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

875S Sheldon C. Sommers, M.D. This project was extended for the month of October 1973 in a pro rata amount of $7,539.50 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

877S Herbert B. Herscowitz, Ph.D. This project was extended for the month of October 1973 in a pro rata amount of $2,719.00 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

The following authorizations were approved:

Contract 2-E Microbiological Associates Incorporated. $115.10 This amount was expended for testing five available inbred lines at Lakeview Hamster Colony for different aryl hydrocarbon hydroxylase levels. This contract was approved for $157,000.00 at the Planning Committee meeting on December 8, 1972. Of this amount, a pro rata award of $25,000.00 was authorized covering the period February 1 through March 31, 1973. The total contract as finally signed was for $150,000.00 covering the period February 1, 1973 through January 31, 1974.

Contract 17 William E. Benedict, M.D. While traveling on contract business, Dr. Benedict incurred expenses totaling $548.92 and was reimbursed.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

MEETING

EXECUTIVE COMMITTEE OF THE SCIENTIFIC ADVISORY BOARD

JUNE 13, 1973

NEW YORK, N. Y.

Attendance

Sheldon C. Sommers, Chairman
Richard J. Bing
John P. Wyatt
William U. Gardner ex officio
Robert C. Hockett, Acting Scientific Director
F. W. Nordsiek, Associate Scientific Director
H. H. Ramm
W. T. Hoyt

Requests for Funds

1. The following requests for funds were recommended for approval subject to the determination by the Acting Scientific Director as to priority and the amount to be funded:

573-BR1S
573-BR2S — Clayton G. Loosli, Ph.D., M.D. $36,662.00 per quarter for the twelve-month period beginning July 1, 1973, or an amount of approximately $9,000.00 per quarter more than the present awards.

703-B — Albert H. Niden, M.D. $15,000.00 Approved for the seven months from June 1, 1973 through December 31, 1973, following his transfer from Temple University to Drew Postgraduate Medical School, of which amount $4,000.00 represents unexpended funds released by Temple University.

734R2S — Carroll E. Cross, M.D. $4,500.00 For the completion of the current cigarette smoke exposure studies and preparation of protocols for future studies. Such funds, however, should not imply any support in the future.

766-AS — Richard A. Lerner, M.D. $5,000.00

776-A — Elliot S. Vesell, M.D. $25,000.00 For the completion of a project to determine the extent to which individuals vary in rates of nicotine elimination, provided the Acting Scientific Director makes a site visit and is satisfied that the radioimmunoassay being used works satisfactorily.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Contract 16 — Richard A. Lerner, M.D. $39,000.00 as a service contract for one year in connection with the Microbiological Associates Incorporated contracts.

Contract 17 — William E. Benedict, M.D. $10,000.00 as a service contract for one year in connection with the Microbiological Associates Incorporated contracts.

2. The following requests for funds were rated for disapproval:

Attallah Kappas, M.D. A request for an unstated amount of interim funds to support the salaries of two investigators.

Stanley Schachter $6,000-7,000 to defray expenses for conducting a conference of biologists and psychologists.

The Wistar Institute. $10,605.00 requested for interim support for salaries of investigators who had been working with Dr. Watanabe covering the time between the expiration of grant 782R1 and the presentation of an application for continuation.

3. Subsequent to the meeting, of the requests recommended for approval and referred to in (1), the Acting Scientific Director determined that the following should be approved in the respective amounts indicated:

573-BR1S — Clayton G. Loosli, Ph.D., M.D. $9,000.00 July 1 through September 30, 1973.

573-BR2S — $111,150.75 covering the nine-month period from October 1, 1973 through June 30, 1974. The action supersedes the $84,000.00 award indicated in the minutes of the March 1973 Scientific Advisory Board meeting.

703-B — Albert H. Niden, M.D. $15,000.00

734R2S — Carroll E. Cross, M.D. $4,500.00

766-AS — Richard A. Lerner, M.D. $5,000.00

776-A — Elliot S. Vesell, M.D. $8,500.00 as a supplement to the current project 776M2R1, with a time extension from June 1 to November 1, 1973. A modified application for continuation (776-AM) is to be submitted for consideration by the Scientific Advisory Board in October, with an up-to-the-minute report of progress.

Contract 16 — Richard A. Lerner, M.D. $39,000.00

Contract 17 — William E. Benedict, M.D. $10,000.00

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

4. Twenty-two inquiries suggesting outlines of possible project proposals were considered. Of this number, five were rated sufficiently interesting and appropriate to warrant encouraging formal applications. Four were deferred in order to obtain more specific information.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

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LIST OF APPLICATIONS

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

APPLICATIONS TO BE CONSIDERED

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
CANCER				
904	Apirion	$50,496		Andervont, Gardner, Huebner, and Meier
922	Arcos	$49,738		Andervont, Huebner and Meier
920	Benedict	$25,700		Andervont, Gardner, and Huebner
935	Fishman	$62,853		Andervont, Huebner, and Jacobson
877R1	Herscowitz	$29,940	$32,623	Andervont, Gardner, and Huebner
924	Houck	$36,098		Andervont, Gardner, and Huebner
875R1	Ioachim (formerly Sommers)	$79,815	$90,474	Gardner, Huebner, and Loosli
603C	Kauffman	$30,521 (plus ca $7,500)	$25,000	Andervont, Gardner, Wyatt, and Sommers
572C	Levy	$49,119	$20,000 (6 mos.)	Andervont, Gardner, and Huebner
891R1	Neurath	$44,850	$41,000	Gardner, Huebner, and Meier
925	Rao	3,950 Pounds		Andervont, Gardner, and Jacobson
931	Rosen	$12,157		Andervont, Gardner, and Huebner
709CR1	Severi	$19,948	$15,000	Andervont, Gardner, and Huebner
933	Shaw	$76,721		Andervont, Gardner, Huebner, and Loosli
930	Sivak	$54,838		Andervont, Huebner, and Jacobson
934	Stanley	$65,420		Andervont, Huebner, and Jacobson

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Applications

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
PULMONARY				
915	Bresnick	$40,650		Andervont, Gardner, and Sommers
906	Brooks	$14,658		Loosli, Sommers, and Wyatt
937	Cross	$44,650		Gardner, Jacobson, Sommers, and Wyatt
919	Daniels	$51,216		Loosli, Meier, and Wyatt
815B	Esterly	$34,482	$30,028	Loosli, Sommers, and Wyatt
921	Finley	$90,712		Gardner, Loosli, and Wyatt
916	Goldstein, R.	$29,900		Loosli, Sommers, and Wyatt
878R1	Hamosh	$28,265	$26,870	Bing, Loosli, Sommers, and Wyatt
914	Lamm	$34,672		Jacobson, Loosli, and Wyatt
741B	Lauweryns	$24,556	$21,090	Gardner, Loosli, and Wyatt
908	Leeds	$56,485		Loosli, Sommers, and Wyatt
907	Mandl	$38,717		Loosli, Sommers, and Wyatt
841A	Mittman	$36,608	$35,000	Jacobson, Loosli, and Wyatt
702C	Niden	$25,618	$15,000 (7 mos.)	Loosli, Sommers, and Wyatt
928	Rifkin	$36,077		Jacobson, Loosli, and Wyatt
913	Sbarra	$65,100		Bing, Loosli, and Wyatt
936	Vidic	$40,738		Bing, Loosli, Sommers and Wyatt

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Applications

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
CARDIOVASCULAR				
843R2	Barger	$50,000	$50,000	Bing, Meier, and Sommers
926	Bhagat	$31,360		Bing, Jacobson, and Sommers
918	Downey	$15,290		Bing, Jacobson, and Wyatt
839R2	Fisher	$24,978	$25,300	Bing, Meier, and Sommers
910	Ramsey	$10,859		Bing, Gardner, and Jacobson
889R1	Regan	$45,500	$44,776	Bing, Gardner, and Sommers
814R2	Ryan (Una Smith)	$35,373	$32,908	Bing, Loosli, Sommers, and Wyatt
864A	Slotkin	$13,346	$12,110	Bing, Gardner, and Loosli
833A	Weltman	$58,386	$42,663	Bing, Jacobson, and Sommers

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Applications

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
PHARMACOLOGY				
923	Boden	$36,206		Bing, Gardner, and Jacobson
905	Domino	$52,885		Gardner, Jacobson, and Meier
836AR1	Essman	$59,370	$40,000	Bing, Jacobson, and Meier
642C	Goldstein, L.	$33,350	$29,000	Bing, Jacobson, and Meier
917	Hudgins	$22,400		Bing, Gardner, and Meier
929	Leete	$23,709		Gardner, Jacobson, and Sommers
868R1	McKennis	$74,184	$60,000	Bing, Gardner, and Jacobson
909	Rosecrans	$20,260		Gardner, Meier, and Sommers
869R1	Rubin	$16,675	$21,300	Gardner, Meier, and Sommers
467C	Westfall	$21,953	$33,000	Bing, Gardner, and Jacobson
927	Wilson	$ 8,973		Gardner, Jacobson, and Sommers
MISCELLANEOUS				
787B	Friedman	$99,440	$100,000	Gardner, Jacobson, Loosli, and Sommers
932	Heinrichs	$73,108		Bing, Loosli, and Sommers
912	McClugage	$38,094		Bing, Jacobson, and Meier

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CANCER

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#904 - APIRION

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 9, 1973

Grant Application No. 904

To: The committee comprising Drs. Andervont, Gardner and Huebner

Subject: David Apirion, Ph.D., Washington University, St. Louis
New application No. 904
"Alterations in Nuclei Associated with Malignancy"

History

This proposal was Case #123, and application was discouraged for lack of relevance to CTR objectives. We telephoned Dr. Apirion when he submitted; he stated that he could not defend CTR program relevance, but hoped that the Scientific Advisory Board might see such.

Application No. 904 requests $50,496 plus two additional years.

Documents Submitted

Attached is application dated February 22, 1973.

Reprints of the five publications listed on page 14 have been provided, and will be forwarded if you so request.

Comment

Dr. Apirion seems to be trying to orient his basic scientific interests towards cancer. As page 4 of the application indicates, he currently has two National Science Foundation grants.

Page 4 also shows he has applied to three cancer research agencies. I have determined the outcome as follows:

American Cancer Society: Approved, not funded. (This was a renewal application.)

Damon Runyon Fund: Disapproved because of low priority.

National Cancer Institute: Approved, but funding unlikely.

F.W.N.

FWN:wg
Encl.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

#904

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N.Y. 10022

MAR 8 1973

RECEIVED

Application For Research Grant

Date: February 22, 1973

1. Name of Investigator(s): (include Title and Degrees)

David Apirion, Ph.D.
Associate Professor of Microbiology

2. Institution & Address:

Washington University
Lindell & Skinker
St. Louis, Missouri 63130

3. Short Title of Project:

Alterations in nuclei associated with malignancy

4. Proposed Starting Date: 1 July 1973

5. Anticipated Duration of this Specific Study: 30 June 1976

6. Brief Descripton of Objectives or Specific Aims:

1) The development of a two dimensional gel electrophoresis system amenable to autoradiography for the analysis of chromosomal proteins.

2) Comparison of total chromosomal proteins in transformed and non-transformed lines. This will be tested in material isolated from cromatin, from total metaphase chromosomes and from single chromosomes.

3) Comparison of acetylation phosphorylation and other modifications of chromosomal proteins in transformed and nontransformed lines. This will be tested in material isolated from chromatin, total metaphase chromosomes and from single chromosomes.

4) Isolation of differentiation variants and analysis of their chromosomal proteins.

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7. Give a Brief Statement of your Working Hypothesis: The behaviour of a malignant cell, for instance unrestricted growth, is due to changes in the response of its genome to environmental (cellular) stimuli. The capacity of the genome to respond to these stimuli depends to a large extent on nuclear proteins.

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

8. *Details of Experimental Design and Procedures: (Attach Separate Pages)*

(See pages 5-11.)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

General facilities at our disposal include controlled temperature rooms (30°, 37°), media room, autoclaves, cold rooms (4°, -18°), and a -70° refrigerator. Preparative and analytical ultracentrifuges with a number of rotors. Amino acid analyzer. A scintillation and a dry counter for radioisotopes. High voltage electrophoretic apparatus. Spacious lab area and office. A number of water baths and incubators, small centrifuges, and various other facilities necessary in a modern biochemical laboratory. We have excellent animal quarters, and fairly adequate facilities for tissue culture work. Spacious lab space is available.

10. Additional Requirements:

Most of our present tissue culture facilities are concentrated in a central laboratory which is next to my lab and serves all the department. Therefore the initial work can be started even without addition of any new equipment; however, for the continuation of the project facilities for tissue culture work would have to be introduced into my laboratory.

11. Biographical sketches of all principal and professional personnel (append)

(See pages 12 & 13.)

12. List of publications: (Five most recent as pertinent) (append)

(See page 14.)

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

R: REDACTED MATERIAL

13. Budget (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
David Apirion		
Ruth Kaplan		
Technical		REDACTED
Edward Watson		
Vera Johnson		
Ernestine Love		
Fringe Benefits (Social Security)		
	Sub-Total	
B. Consumable Supplies (list by categories)		
Liquid Nitrogen		400
Tissue Culture Medium		2,200
Chemical Substrates and Enzymes		1,450
Radioisotopes		1,650
Glassware and Plasticware		1,100
Animals and animal supplies		600
	Sub-Total	7,400
C. Other Expenses (itemize)		
National Scientific meeting for two persons		700
Publications costs (page charges, illustration, reprints, typing)		300
Books and Journals		100
Equipment Maintenance Contracts		500
	Sub-Total	1,600
D. Permanent Equipment (itemize)		
Water Bath for Spinners with Magnetic Rotors		1,000
Laminar Flow Hood		1,500
Inverted Microscope		1,200
Electrophoresis Units (2)		800
Power Supply Units (2)		1,600
		6100
E. Overhead (15% of A+B+C)		5,791
	Total	$50,496

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	31,381	7,750	1,800	3,500	6,140	$50,571
Year 3	33,264	8,000	2,000	2,400	6,490	$52,154

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature David Apirion
Director of Project (314) 367-6400 Ext. 3451 Telephone

Signature Joe F. Evans
Business Officer of the Institution Joe F. Evans
(314) 863-0100, Ext. 4570 Telephone

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

	Title of Project	Source	Amount	Duration
Current				
	Genetic and Structural Studies of E. coli Ribosomes	National Science Foundation	$ 32,346	5/15/72-5/14/73
	A Biological Approach to Controlled Synthesis of Desired Proteins	National Science Foundation	16,135	11/1/72-4/30/74
Pending				
	Studies with Neuroblastoma Lines	American Cancer Society	109,978	7/1/73-6/30/75
	Chromosomal Proteins in Normal and Malignant Cells	Damon Runyon Memorial Fund for Cancer Research Inc.	89,446	7/1/73-6/30/75
	The Fate of RNA in Malignant and Normal Cells	National Institutes of Health	151,542	9/1/73-8/31/76

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Introduction. A malignant cell can be viewed as a cell which has lost part of its regulatory capacity, a cell which ceases to respond to various stimuli as does the precursor cell.

Parts of the genome of a mammalian cell seem to be permanently blocked, while some other parts seem to be capable of responding to stimuli in the cellular environment. Cells from different tissues in the organism or in tissue culture can respond to similar effector molecules, such as cAMP. However, their responses vary: fibroblast cells and neuroblastoma cells both respond to cAMP but very different proteins are produced and different processes apparently occur in both cells during these responses. That part of the genome which responds to the effector must be determined by something in the cell. Since in all likelihood genomes of different tissues, in the same organism, are by and large identical, the differences among nuclei from different tissues could be due to chromosomal proteins.

Since the genome of a malignant cell ceases to behave as does a genome of the parental nontransformed cell it is quite likely that a change occurred in the chromosomal proteins.

There is a growing body of evidence linking changes in the genome with specific cancers; see for instance references 1 - 3. However, these changes do not seem to be too specific, i.e. it is not clear whether the genome is altered or whether the change could be related to alterations in chromosomal proteins. A much more interesting observation is that the synthesis of nuclear acidic proteins is increased after SV40 infection (4). In order to analyze such situations (transformed versus nontransformed cells) we wish first to develop proper techniques for qualitative and quantitative analysis of chromosomal proteins and to use these techniques on specific cell lines where the analysis could be most informative.

Materials. We plan to work exclusively with mouse cells. In order to be able to isolate specific chromosomes (see below) we wish to use a specific mouse line that contains only one metacentric chromosome. Such a strain was isolated by crossing Mus musculus to Mus poschiavinus and back crossing the hybrid to the first parent (5). (Mus poschiavinus contains seven metacentric chromosomes.) The single metacentric chromosome in the strain isolated from the back cross is distinct and it replaces two acrocentric musculus chromosomes. In all likelihood it should be feasible to separate this chromosome from all others. Other mouse lines are available in which by reciprocal translocations between two acrocentric chromosomes one larger chromosome and one smaller chromosome have been produced. In such a line it might be possible to separate individually two chromosomes, the large and the small, from all other chromosomes. Separation will have to be carried out on cells grown in tissue culture arrested in the metaphase stage.

Isolation of chromatin, chromosomes and their analysis. Chromatin and chromosomes would be isolated from cells transformed by polyoma virus or other means and nontransformed cells, and from cells during the process of cell transformation (chromatin). (Techniques for such isolations are available and some of the references are mentioned above.) As chromosomes are separated

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the cells would be labeled first (long term) with ^{14}C-amino acids in order to label the chromosomal proteins; thus even very small quantities of material could be analyzed by autoradiography.

Prior to the isolation of the chromatin and chromosomes we shall develop two dimensional polyacrylamide gel electrophoresis for the analysis of the chromosomal proteins. At present this technique developed by Kaltschmidt and Wittmann (6), is probably the most sensitive technique available for identification of proteins in a mixture. Moreover ribosomal proteins and chromosomal proteins both share common characteristics, especially with respect to solubility, and therefore it sould be quite easy to adapt the existing techniques to the analysis of both the histones and the acidic proteins. For further details about the analysis, see below.

The qualitative and quantitative pattern of the chromosomal proteins in the chromatin and chromosomes isolated from transformed and nontransformed cells, would be compared. This analysis would be augmented by proper in vivo labelling of the proteins with radioisotopes. Thus we should be able to determine whether or not there is a unique pattern to chromosomal proteins during cell infection with a virus, during the establishment of transformation and after the process is finished and the condition is maintained. Similar analysis would be carried out on cells transformed by other means, e.g. 3T3 mouse cells and cells derived from them which have lost contact inhibition.

Acetylation and phosphorylation of chromosomal proteins. Chromosomal proteins, and specifically acidic proteins, are known to undergo acetylation and phosphorylation which seems to be relevant to either the cell cycle or to regulation of gene function. These parameters would be measured on total chromosomes, chromatin and isolated chromosomes. This would be done during regular growth and after infecting cells with the virus. In a different approach chromosomal isolated chromosomes and the isolated proteins would be used as substrates to measure acceptance of phosphate by protein phospho kinase mediated by cAMP.

Such comparisons as described in the last two sections coupled with knowledge about gene activity of a specific chromosome (see below) would lead to meaningful correlations between gene activity or gene restriction and transformation. We should be able to determine whether there is any indication that the programming written into the cells during differentiation is changed during and after transformation.

Variants of Differentiation. A very interesting situation that ties together a viral infection and differentiation is the cloned line of murine proerythroblastoid cells. Such a line can be transformed by infecting it with Friend leukemia virus (7, 8). Such transformed cells can be made to differentiate by addition of dimethylsulfoxide (Me_2SO) to the culture medium (9).

In this system the comparison of chromosomal proteins, their phosphorylation and acetylation, can be made among the three stages - the uninfected cells, the infected transformed cells and the differentiated cells. Since recently it was shown that during this differentiation process a specific messenger is produced (globin mRNA; 10) it should be feasible to find out what specific changes in the nucleus, in chromosomal proteins, were caused during this viral

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the cells would be labeled first (long term) with ^{14}C-amino acids in order dependent differentiation process.

This phenomenon mentioned above suggests that it should be possible to study nuclear changes during transitions from one differentiated stage to another. Another case that we shall study is the transition in neuroblastoma cells from undifferentiated to differentiated cells, which can be caused by small effector molecules such as cAMP (11).

It should be possible to isolate from existing cell lines variants that can produce proteins which are not usually produced. For instance, a cell line developed from a fibroblast such as a 3T3 line does not produce globin or liver proteins such as serum albumin. However it has been shown by Petterson and Weiss (12) that hybridization of a mouse fibroblast line with a rat hepatoma line gave a hybrid which started producing mouse serum proteins. Therefore we should select lines that produce new products, with and without transforming the line with a virus. First, we shall find out whether the frequency with which such new variants, referred to herein as differentiation mutants, can be increased after transformation. If this is the case it would suggest that changes in the genome which led to transformation and malignancy are relevant to changes in the genome which lead to differentiation. A number of agents, such as alkylating agents, nitrous acid, acridines, acridine half mustards, etc., would be used to induce such variants, and from finding out which of them is effective some insight could be gained toward what causes this process.

There are a number of possible ways to score or select for such variants, (differentiation mutants) of which we mention here only a few.

1) Selection for the production of an enzyme that can be stained for histochemically,
2) Selection for heme production (stain with benzidin), or autoradiography with iron,
3) Selection for serum albumin producing cells.

1) The enzyme acetyl cholineesterase: Nirenberg and his coworkers (13) showed that it is possible to stain neuroblastoma colonies for this enzyme. Therefore 3T3 cells would be mutagenized or treated in various ways, e.g., with DMSO, and colonies would be produced and stained for production of the enzyme acetyl cholineesterase. Since this is a visible reaction it should be feasible to screen a large number of colonies in this assay. Should such variants be found, the process would be repeated under conditions where each colony is grown in duplicate, so that further analysis of any suspect colony (which probably would be killed by the staining procedure) could be continued. Growing of large number of colonies in an ordered fashion is possible in multiwelled plates (see ref. 14). We have ascertained that we can grow mouse L cells in such units.

2) Detection of heme producing lines: this could be accomplished either by staining with benzidin, or by autoradiography with iron (15), using techniques similar to those developed by Milcarek and Weiss (16) for the isolation of DNAseless mutants of E. coli. We are at present isolating RNAseless mutants of E. coli by this technique, and are satisfied with it. I am confident that it is a very adequate technique for the isolation of variants

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that might show a change in incorporation of a specific radioisotope.

3) Isolation of serum albumin producing cells from a 3T3 fibroblast line: For this purpose an antibody against purified mouse serum albumin would be prepared, (excellent facilities are available in our department for this purpose) and colonies arising from treated (mutagenized) cells would be treated with the antibody in the manner with which Sharff and colleagues (17) treat colonies to find out whether or not they produce antibodies.

There is the possibility that the serum protein is synthesized but not secreted, and is thus not detected by the antibody. If after screening a reasonable number of colonies (about 10,000) none producing serum albumin that should be detected, treated (mutagenized) cells would be inoculated into the small wells. The beauty of these multiwell plates is that they are treated in or as test tubes and therefore we shall open the cells grown in them and then treat them with the antibody in agar. Thus a precipitate could be formed and observed. Again, if this fails, the antibody can be labeled by an iodine radioisotope and an antigen antibody complex can be screened for by autoradiographic techniques.

I would like to emphasize that the three characteristics described here are only examples, and many other phenotypes can be screened for. Therefore I have no doubt that the isolation of "differentiation variants" is feasible and the analysis of the chromosomal proteins and their chemical modification in such lines should be very illuminating and very critical for the understanding of cell differentiation. I would like also to point out that in these screenings or selections, we shall be looking for positive traits and therefore even if the colonies we analyze are mixed clones arising from some modified and some unmodified cells, we should be able to discover these clones, since they should score as positive or partial positive in the tests.

Isolation and analysis of specific chromosomes. There are two main aims in the analysis of unique chromosomes; 1) concentration on a unique chromosome some of the active genes of which might be known and determining specifically whether there are any quantitative or qualitative changes or modifications in its proteins, during or after the process of cell transformation, or in differentiation variants. 2) Location of similar parameters about a chromosome to which the virus actually attached. While it probably would be desirable to know the latter, knowing the former would be helpful to determine whether specific changes occur in the nucleus (chromosomes) after cell transformation. Although the isolation of a specific chromosome and its analysis may be achieved it cannot, of course, be predicted that the virus would attach specifically to the chromosome that we plan to isolate. However, this might be achieved by using the mouse man or mouse-Chinese hamster cell-cell hybridization.

In a hybrid cell line of mouse and man (18), using a human viral transformed line, the human chromosomes would carry the SV40 viral genome. In such a line it should be possible to isolate clones that lost most of the human chromosomes. It should be also possible to hybridize a mouse transformed cell with a human nontransformed cell or Chinese hamster cell and to isolate hybrids that carry small numbers of the mouse chromosomes. A hybrid line would be searched for that carries just a single human or mouse chromosome (the mouse chromosome should be relatively easy to identify) and which still carries the virus (this can be verified by analysis

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of specific viral antigens). While the hybrids would usually lose preferentially the human chromosomes, it should be possible by irradiation of the mouse cells prior to the hybridization, to cause preferential loss of mouse chromosomes (19). Perhaps among such specific hybrids there will be one from which the mouse chromosome could be separated from all the others. This should allow us to analyze the chromosomal proteins of a chromosome that carries a viral genome.

It is clear that this project has a number of experimental approaches, and success in any of them would make our efforts worthwhile and very rewarding. The independent parts of the project are the following: a) Development of two dimensional gel electrophoresis for chromosomal proteins, b) Autoradiography for acetylation and phosphorylation of chromosomal proteins; this depends on a but can be done independently by one dimension gel electrophoresis. c) Isolation of a single chromosome from a mouse, d) Isolation of differentiation variants, e) Isolation of a chromosome carrying a viral genome.

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7. Friend, C., Patuleia, M.C., and deHarven, E. (1966). Nat. Cancer Inst. Monogr. 22:505-520.
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19. Pontecorvo, G. (1971). Induction of directional chromosome elimination in somatic cell hybrids. Nature 230:367-369.

Work and experience relevant to this project

For my master and Ph.D. thesis I worked on genetics of higher organisms (20-25) which gives me a basic familiarity with genetic problems of eukaryotic cells. During my studies for the Ph.D. I developed a two-way selection technique (see ref. 2). Similar principles are used now in the selection of resistant mutants in tissue culture which become simultaneously auxotrophic. Also I had the benefit of working with Professor G. Pontecorvo (1961-63) at the time in which he initiated somatic cell genetics in tissue culture. During my studies for the Master's degree I prepared and taught cytogenetic laboratories which should help me now with the chromosomal aspects of the project. Also this year I taught a graduate course on eukaryotic chromosomes, which has further orientated me towards this research problem. My experience with ribosomal proteins (25-27) would, I believe, be most useful since the techniques we use to analyze ribosomal proteins are most appropriate to the analysis of chromosomal proteins as in both cases one is dealing with complex organelles which are composed of nucleic acids and proteins.

For analysis of ribosomal proteins we use a variety of polyacrylamide gels, round gels, one dimension slab gels and two dimension gels (the first dimension of electrophoresis is done in a round gel and the second is in a slab gel). Also recently we introduced thin (1.5mm) one dimensional slab gels which are adequate for autoradiography, since they can be easily dried. We have already made some preliminary trials with nuclear proteins isolated from chromatin of mouse liver cells and mouse L-cells using the thin layer one dimensional slab gels. It is clear from the results that before long we should have at our disposal very powerful tools for the analysis of chromosomal proteins. We chose to develop a technique amenable to autoradiography for two reasons: 1) we anticipate that most of the work on proteins isolated from chromosomes would have to be carried out on very small amounts of material where identification would have to depend

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on radioisotopes; 2) an important part of the analysis of chromosomal proteins would involve modifications that are taking place in them during a variety of cellular events related to transformation and malignancy. Such physiological modifications can be detected by proper radioisotope labeling.

In order to learn cell hybridization techniques I spent during 1971 a number of weeks in Dr. F. Ruddle's laboratory (Yale University) who is one of the leaders in this field. In all the specific areas which are outside my expertise there are a number of people in the Medical School who are very competent in these areas and who have promised help and counsel if necessary.

PERTINENT REFERENCES

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22. Apirion, D. (1963). Formal and physiological genetics of ascospore colour mutants in Aspergillus nidulans. Genet. Res. 4: 276-283.

23. Apirion, D. (1965). The two-way selection of mutants and revertants in respect of acetate utilization and resistance to fluoo-acetate in Aspergillus nidulans. Genet. Res. 317-329.

24. Apirion, D. (1966). Recessive mutants at unlinked loci which complement in diploids but not in heterokaryons of Aspergillus nidulans. Genetics, 53:935-941.

25. Krembel, J., and Apirion, D. (1968). Changes in ribosomal proteins associated with mutants in a locus that affects Escherichia coli ribosomes. J. Mol. Biol. 33:363-368.

26. Deusser, E., Stoffler, G., Wittmann, H.G., and Apirion, D. (1970). Ribosomal proteins XVI. Alteres S4 proteins in Escherichia coli revertants from streptomycin dependence to independence. Molec. Gen. Genetics, 109: 298-302.

27. Stoffler, G., Deusser, E., Wittmann, H.G., and Apirion, D. (1971). Ribosomal proteins XIX. Altered S5 ribosomal protein in an Escherichia coli revertant from streptomycin dependence to independence. Molec. Gen. Genetics, 111: 334-341.

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R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: David Apirion SOC. SEC. NO. R

DATE & PLACE OF BIRTH: REDACTED

CITIZENSHIP: R

MARITAL STATUS: REDACTED

EDUCATION:

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Glasgow University, Glasgow, Scotland	Ph.D.	1960-63	Biology Genetics

FIELD OF SPECIALIZATION: Molecular Biology and cellular organization.

PRESENT POSITION:

Associate Professor, Washington University School of Medicine, Department of Microbiology, St. Louis, Missouri — 1970 to present

PROFESSIONAL EXPERIENCE AND APPOINTMENTS:

Research and teaching assistant, Hebrew University, Jerusalem, Israel	1959-60
Research Student, Glasgow University, Glasgow, Scotland	1960-62
Assistant Lecturer, Glasgow University, Glasgow, Scotland	1962-63
Research Fellow, Harvard University, Cambridge, Massachusetts	1963-65
Assistant Professor, Washington University, Department of Microbiology, St. Louis, Missouri	1965-70

HONORS:

The Tuvia Kushnir Prize	1960
The Alexander Milman Prize	1961
The Sir Maurice Bloch Award	1962

SOCIETY MEMBERSHIPS:

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PUBLICATIONS

David Apirion

M.S. Thesis (1960) Distribution of albino genes in populations of Dactylis glomerate, in Israel.

Ph.D. Thesis (1963) Studies on the selection of mutants in Aspergillus nidulans.

1. Apirion, D., and Zohary, D. Chlorophyll lethal in natural populations of the orchard grass (Dactylis glomerate L.) A case of balanced polymorphism in plants. Genetics 46: 393-399 (1961).

2. Apirion, D. A general system for the automatic selection of auxotrophs from prototrophs and vice versa in microorganisms. Nature 195: 959-961 (1962).

3. Apirion, D. Formal and physiological genetics of ascospore colour in Aspergillus nidulans. Genetics Res. 4: 276-283 (1963).

4. Apirion, D. The two-way selection of mutants and revertants in respect of acetate utilization and resistance to fluoro-acetate in Aspergillus nidulans. Genetics Res. 6: 317-329 (1965).

5. Apirion, D. Recessive mutants at unlinked loci which complement in diploids but not in heterokaryons of Aspergillus nidulans. Genetics 53: 935-941 (1966).

6. Apirion, D. Altered ribosomes in a suppressor strain of Escherichia coli. J. Mol. Biol. 16: 285-301 (1966).

7. Mangiarotti, G., Apirion, D., and Schlessinger, D. Selection of sucrose-dependent Escherichia coli to obtain envelope mutants and fragile cultures. Science 153: 892-894 (1966).

8. Apirion, D. A new ribosomal gene affecting the 50S subunit in Escherichia coli. Cold Spring Harb. Symp. Quant. Biol. 31: 630-631 (1966).

9. Schlessinger, D., and Apirion, D. Differences in the protein synthesizing machinery of cells of Escherichia coli grown aerobically and anaerobically. Cold Spring Harb. Symp. Quant. Biol. 31: 680-681 (1966).

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57. Simon, M., and Apirion, D. Increased inactivation of messengers in Escherichia coli temperature sensitive mutant. Nature New Biology 239: 79-81 (1972).

58. Weatherford, S.C., Wright, M., and Apirion, D. Ribonuclease II activity in a "presumptive" RNase II deficient mutant. J. Bacteriol. 111: 835-837 (1972).

59. Simon, M., and Apirion, D. A differential effect of adenosine 3',5'-cyclic monophosphate on the synthesis of functional enzymes from two different cistrons of the lac operon. Biochem. Biophys. Res. Commun. 48:1168-1172 (1972).

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R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: Ruth Kaplan Name before Marriage:

DATE AND PLACE OF BIRTH: REDACTED

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EDUCATION: REDACTED

FIELD OF SPECIALIZATION: Molecular Biology

PROFESSIONAL EXPERIENCE AND APPOINTMENTS:

Research Assistant, Hebrew University, Hadassah Medical School, Jerusalem, Israel	1956-1957
Ph.D. Student and Teaching Assistant, Hebrew University, Hadassah Medical School, Jerusalem, Israel	1958-1962
Instructor, Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel	1963-1966
Lecturer, Department of Biochemistry, Tel-Aviv University, Israel	1967-1971

PRESENT POSITION:

Visiting Research Associate, Washington University School of Medicine, Dept. of Microbiology, St. Louis, Missouri 1971-

SOCIETY MEMBERSHIPS: REDACTED

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12. List of five pertinent publications (reprints enclosed)

1. Deusser, E., Stoffler, G., Wittmann, H. G., and Apirion, D. Ribosomal proteins: XVI. Altered S4 proteins in Escherichia coli revertants from streptomycin dependence to independence. Molec. Gen. Genetics, 109: 298-302 (1970).

2. Stoffler, G., Deusser, E., Wittmann, H. G., and Apirion, D. Ribosomal proteins. XIX. Altered S5 ribosomal protein in an Escherichia coli revertant from streptomycin dependence to independence. Molec. Gen. Genetics 111:334-341 (1971).

3. Simon, M., and Apirion, D. A messenger RNA from the lactose operon of Escherichia coli that can not direct the production of functional β-galactosidase in absence of exogenous adenosine 3'-5'-cyclic monophosphate. Genetics, 7: 1-18 (1972).

4. Simon, M., and Apirion, D. A differential effect of adenosine 3',5'-cyclic monophosphate on the synthesis of functional enzymes from two different cistrons of the lac operon. Biochem. Biophys. Res. Commun. 48: 1168-1172 (1972).

5. Meyhack, B., Meyhack, I., and Apirion, D. Colicin E3: A unique endoribonuclease. Proc. Natl. Acad. Sci. U.S.A. 70: 156-160 (1973).

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#922 - ARCOS

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 17, 1973

Grant application No. 922

TO: The committee comprising Drs. Andervont, Huebner and Meier

SUBJECT: Joseph C. Arcos, M.D., Tulane University, New Orleans
New application No. 922
"Synergistic effects of polycyclic hydrocarbons and nitrosamines..."

History

During May an informal inquiry was received from Drs. Arcos and Argus. In view of manifest relevance to the CTR program, staff decided to offer these investigators the option of applying forthwith (with no implication of commitment).

At the same time copies of the inquiry were sent to the Executive Committee. Dr. Bing responded, saying "...I find it interesting but extremely expensive. I do not believe it to be quite deserving of this tremendous outlay of money..."

Application #922 asks for $49,738. plus three additional years. The request for a fourth year is defended by the applicant in a footnote on page 1 of the application. The amount now requested is more than the initial estimate of $128,000. total direct costs for four years, on which Dr. Bing commented.

Documents Submitted

1. Attached is application dated 7/9/73 (13 pages).
2. Also attached are CV's of Dr. Arcos (16 pages), Dr. Argus (12 pages), and Dr. Simon (9 pages).
3. Reprints have been provided of papers numbered as follows in the CV of Dr. Arcos: 29, 45, 47, 49, 54, 56, 59, 62, 64, 66. Any of these will be forwarded if you so request.

Comment

Dr. Gardner has not been asked to serve on this committee in the hope of reducing his current load; but his comment would be most pertinent.

FWN

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Attachments

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Drs. Andervont
Huebner
Meier

· CARCINOGENESIS ·

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8887

JUL 16 1973

Application for Research Grant
(Use extra pages as needed)

Date: 7/9/73

1. Principal Investigator (give title and degrees):

Joseph C. Arcos
Professor of Medicine (Biochemistry)
L. Chem., Ch. E., D. Sc.

Mailing address:

Seamen's Memorial
Research Laboratory
USPHS Hospital
210 State Street
New Orleans, La. 70118

2. Institution & address:

Tulane University School of Medicine
1430 Tulane Ave.
New Orleans, La. 70112

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

4. Short title of study: Synergistic effects of polycyclic hydrocarbons and nitrosamines in pulmonary carcinogenesis. Potential repressors of metabolic activation of nitrosamines.

5. Proposed starting date: 1/1/74

6. Estimated time to complete: 4 years *

7. Brief description of specific research aims:

First, to explore experimentally the very definite possibility (see 8. Background and brief statement of working hypothesis) that a significant proportion of the lung tumor incidence of cigarette smokers is due to synergism between hydrocarbons and nitrosamines present in the smoke rather than to the hydrocarbons alone, and that the oral nitrosamine intake of smokers (as dietary nitrosamine contaminants, or pro-nitrosamine additives such as nitrite together with ubiquitously present secondary amines in meat products) could play an important role in their high lung cancer incidence. Second, to investigate

* It is understood that The Council makes renewal awards usually up to 3 years. However, special consideration should be given to the fact that this proposal involves a substantial number of carcinogenesis bioassay systems in which each animal group will have to be maintained for 18 months, followed then by the autopsy of large numbers of animals, histopathology of the tissues, and analysis and organization of the data. Consequently, to keep the experiment manageable, it will be necessary to stagger certain experimental segments within the present study.

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7. continued...

whether certain nontoxic inhibitors of nitrosamine metabolism — a process known to be required for carcinogenesis by these agents — also inhibit nitrosamine carcinogenesis and, hence, would decrease lung tumor incidence by disrupting the hydrocarbon-nitrosamine synergism.

Any bioassay of potential carcinogens or combination(s) thereof must be carried out for a minimum of 1 year, to be meaningful at all, unless a statistically significant percentage of tumor incidence is reached earlier (1); the same holds, evidently, when intending to establish synergistic and co- and anti-carcinogenic effects relative to control group(s) in specific experiments. For this reason it will require a number of years to explore all aspects of the hydrocarbon-nitrosamine carcinogenic synergism in the lung as well as the possible means of disrupting this synergism.

The present proposal represents the first segment of this approach. In this study we will investigate the synergism toward lung tissue of the highly carcinogenic hydrocarbons, 3,4-benzopyrene and 3,4-benzofluoranthene, with the carcinogenic nitrosamines, dimethylnitrosamine, methylethylnitrosamine, and N-nitrosopiperidine — all present in cigarette smoke. In the present study all hydrocarbon-nitrosamine combinations will be given orally, the route of administration by which the first instance of synergism of this type, with a combination of 3-methylcholanthrene and dimethylnitrosamine, was demonstrated in our laboratory (2). Also within the framework of the present study we intend to determine the inhibitory effect of β-naphthoflavone and pregnenolone-16α-carbonitrile on the carcinogenic activity of dimethylnitrosamine; these two agents are powerful inhibitors of dimethylnitrosamine metabolism, and are virtually nontoxic.

8. Background and brief statement of working hypothesis:

A.) Hydrocarbon-nitrosamine synergism. — A previous study (2) in our laboratory has shown that administration of 3-methylcholanthrene inhibits liver tumor induction in rats by simultaneously administered dimethylnitrosamine. In a series of investigations (3-7) we have established that this is due to inhibition in the liver of the biosynthesis of the enzyme, dimethylnitrosamine-demethylase, the key enzyme transforming this nitrosamine to its actual carcinogenic metabolite.

An unexpected and important finding of the carcinogenesis study (2) was that while the hepatocarcinogenicity of dimethylnitrosamine is inhibited by the simultaneous oral administration of 3-methylcholanthrene, the two agents have a powerful synergistic effect in lung tumor induction. Indeed, whereas at the levels used in the study neither the nitrosamine nor the hydrocarbon alone was carcinogenic in the lung, combined administration of the two agents brought about a 15% pulmonary tumor incidence. The practical significance of this lies in the facts (a) that 3-methylcholanthrene is known to be very similar or identical in action to the two potent hydrocarbon carcinogens, 3,4-benzopyrene and 3,4-benzofluoranthene, present in cigarette smoke (8), and (b) that dimethylnitrosamine, methylethylnitrosamine, and N-nitrosopiperidine are also present in the smoke (9) at a

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total level of similar order of magnitude as the hydrocarbons (10). Dimethylnitrosamine and N-nitrosopiperidine are well-known to be potent carcinogens; although methylethylnitrosamine has not been assayed as yet, from what is known about the structure-activity relationships of the dialkylnitrosamines there is no doubt that it will prove to be carcinogenic since both dimethyl- and diethylnitrosamine are potent agents.

The above considerations point to the high probability that a significant proportion of the lung tumor incidence of smokers is due to synergism between carcinogenic hydrocarbons and nitrosamines present in the smoke, rather than to the hydrocarbons alone. Thus, the present approach will open hitherto neglected or unsuspected approaches toward a "safer" cigarette. The working hypothesis has also wider ramifications — to be explored in subsequent phases of this approach — in that a proportion of the lung tumor incidence of smokers could be due, in addition to the hydrocarbons (and nitrosamines) present in the smoke, to particular pattern(s) of diet rich in nitrosamine contaminants or pro-nitrosamine components (i. e., nitrite plus secondary amines).

In the present study administration of both hydrocarbons and nitrosamines will be by oral route to follow the pattern of the previous report (2) which showed that pulmonary synergism does exist between 3-methylcholanthrene and dimethylnitrosamine. In subsequent phases — for approximating more closely the actual human situation — we intend to investigate the synergistic effect of orally administered nitrosamines [and pro-nitrosamine components: nitrite plus secondary amine(s)] with intratracheally instilled hydrocarbons, and then with smoke of known 3, 4-benzopyrene level. Since orally received nitrosamines may also be synergistic with hydrocarbons entering the lung via the trachea, these represent an experimental model exploration of the role of dietary nitrosamines on the lung tumor incidence of smokers. Subsequent phases of this approach will also include exploration of the possible carcinogenesis promotor effect of noncarcinogenic polycyclic hydrocarbons, present in cigarette smoke and found recently to be cocarcinogenic (11), upon pulmonary carcinogenesis by nitrosamines.

A.) Hydrocarbon-nitrosamine synergism — A previous study (2) in our laboratory

B.) Anti-carcinogenic effects of β-naphthoflavone and pregnenolone-16α--carbonitrile. — We have demonstrated that not only polycyclic hydrocarbons but also phenobarbital (3, 4), as well as aminoacetonitrile, β- naphthoflavone and pregnenolone-16α-carbonitrile (7) inhibit the critical metabolic route of dimethylnitrosamine leading to its actual carcinogenic metabolite. Kunz et al. (12) reported that administration of phenobarbital inhibits the carcinogenicity of diethylnitrosamine, and Hadjiolov (13) found that administration of aminoacetonitrile inhibits the carcinogenicity of dimethylnitrosamine.

It is well established that β-naphthoflavone inhibits the carcinogenic action of certain hydrocarbons (e. g., 14, 15). It is unknown as yet whether β-naphthoflavone and pregnenolone-16α-carbonitrile inhibit carcinogenesis by nitrosamines, but from the established evidence that they powerfully inhibit the obligatory metabolic route of dimethylnitrosamine, it is expected that they will be found to be potent inhibitors of nitrosamine carcinogenesis. Since neither of two compounds is carcinogenic, they show definite promise as effective agents to disrupt the hydrocarbon-nitrosamine pulmonary synergism.

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9. Details of experimental design and procedures:

For investigating the synergistic carcinogenic effects toward the lung of 3, 4-benzopyrene and 3, 4-benzofluoranthene, on one hand, and dimethylnitrosamine, methylethylnitrosamine, and N-nitrosopiperidine, on the other, the general conduct of the bioassays will be patterned on our previous study with 3-methylcholanthrene and dimethylnitrosamine (2). The "Synoptic Tabulation of Bioassay Groups" shows the overall experimental design of the synergism experiments and of the experiments on the anti-carcinogenic action of β-naphthoflavone and pregnenolone-16α-carbonitrile.

Hydrocarbon-nitrosamine synergism. — The present experimental design includes certain modifications of the procedure followed in the previous study (2). A minimum of 60 rats serving as negative controls will be maintained parallel with the experimental groups. These rats will receive by stomach tube aqueous methylcellulose (Methocel) solution (the medium used to administer the hydrocarbons in the experimental groups) in the same amount, following the same schedule, and for the same length of time, as the respective experimental groups receiving the hydrocarbons in suspension in that same medium. These negative controls will be divided into 2 or 3 groups and started so as to coincide with the beginning of staggered experimental segments (see Footnote on p. 1). As in the previous study (2) male Sprague-Dawley rats, weighing 180-220 gm initially will be used. We do not plan at this stage to explore the sex-dependence of the synergistic effects studied; however, this will be incorporated in our studies of this phenomenon in subsequent stages of this approach.

3, 4-Benzopyrene and 3, 4-benzofluoranthene will be administered at levels equimolar to 300 μg 3-methylcholanthrene daily 5 times a week (2); this amounts to a daily dose of 282 μg for both hydrocarbons in view of their identical molecular weights. Dimethylnitrosamine will be administered by stomach tube at the same daily dose level (400 μg) as previously (2); however, the length of administration will be extended from 24 to 26 weeks in the expectation of better approximating the TD_{50} dose. The daily doses of methylethylnitrosamine and N-nitrosopiperidine will be equimolar to 400 μg of dimethylnitrosamine; these daily doses are 480 μg and 650 μg, respectively. Methylethylnitrosamine will be administered for 22 weeks for approaching an approximate 50% tumor incidence; this is the best approximation possible for the TD_{50} dose of this nitrosamine, based on an interpolation from some dimethyl- and diethylnitrosamine dose-response data (16, 17). N-Nitrosopiperidine will be administered for 28 weeks; this is based on the dose-response data of Druckrey et al. (18).

The length of administration of both hydrocarbons will run a middle course between the shortest and longest duration of administration of the three nitrosamines studied, i.e., 25 weeks. However, in the groups receiving both a hydrocarbon and a nitrosamine, administration of the hydrocarbon will always be initiated 1 week before the beginning of nitrosamine administration.

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Synoptic Tabulation of Bioassay Groups.

	Compound:	No. of animals:	Daily dose (µg):	Medium of administration:	Length of administration (weeks):
	Negative controls	60	—	Methocel 1%	28
Pulmonary Synergism	3,4-Benzopyrene (BP)	40	282	Methocel 1%	25
	3,4-Benzofluoranthene (BzF)	40	282	— " —	25
	Dimethylnitrosamine (DMN)	40	400	Water	26
	Methylethylnitrosamine (MEN)	40	480	— " —	22
	N-Nitrosopiperidine (NPiP)	40	650	— " —	28
	BP & DMN	40	282 & 400	Methocel & Water	25 & 26
	BP & MEN	40	282 & 480	— " —	25 & 22
	BP & NPiP	40	282 & 650	— " —	25 & 28
	BzF & DMN	40	282 & 400	Methocel & Water	25 & 26
	BzF & MEN	40	282 & 480	— " —	25 & 22
	BzF & NPiP	40	282 & 650	— " —	25 & 28
Inhibition of Carcinogenesis	DMN	40	400	Water	28
	β - Naphthoflavone (NF)	40	20 mg/kg	in diet	29
	Pregnenolone-16α--carbonitrile (PCN)	40	20 mg/kg	in diet	29
	DMN & NF	40	400 µg & 20 mg/kg	Water & diet	28 & 29
	DMN & PCN	40	400 µg & 20 mg/kg	Water & diet	28 & 29

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After the schedules of carcinogen administration will be terminated, the animal groups will be maintained for an additional 11-12 months (2). Following the routine procedure in our laboratory, during this observational period the animals will be checked twice daily and the state of their health carefully followed. During this observational period any animal showing clear evidence of the presence of a tumor (i. e., large subcutaneous or abdominally localized), or morbidity, will be sacrificed, submitted to complete autopsy, and the tissue samples fixed in neutral formaline for histopathology. All survivors will be sacrificed at the end of the observational period and treated as above. Histopathology will be carried out by Dr. Joseph Simon, Professor of Pathology, College of Veterinary Medicine, University of Illinois, Urbana, and by Dr. Cornelia Hoch-Ligeti, Chief, Pathology Service and Research Laboratory, Veterans Administration Center, Martinsburg, W. Va., with whom we have had excellent collaboration for the last 15 years.

Anti-carcinogenic effects of β-naphthoflavone and pregnenolone-16α--carbonitrile. — As it has already been described in "Background and brief statement of working hypothesis" these two compounds are powerful inhibitors of the demethylation of dimethylnitrosamine, the metabolic route which is the controlling step of tumor induction by this agent. Thus, combined administration of either of these two compounds with dimethylnitrosamine should decrease the carcinogenicity of the nitrosamine.

Since both β-naphthoflavone and pregnenolone-16α-carbonitrile are costly compounds, it was necessary to determine first the lowest dietary levels at which saturation of dimethylnitrosamine-demethylase inhibition is reached. In a preliminary study of the dose-dependence of inhibition of dimethylnitrosamine--demethylase biosynthesis by in vivo administered pregnenolone-16α-carbonitrile we found that 22-25 mg/day/kg body weight is slightly beyond the saturation dose for inhibition. This is also the appropriate dose for β-naphthoflavone to reach saturation level, as indicated by a comparison of our preliminary data with the results of Cantrell and Bresnick (19). Thus, in the respective groups the animals will receive β-naphthoflavone and pregnenolone-16α-carbonitrile at a daily dose of 25 mg/day/kg body weight, incorporated in the diet.

To increase the likelihood of a better statistical significance (if our working hypothesis is successful) between the DMN control group and the experimental groups receiving DMN + NF and DMN + PCN, the total dose level of dimethylnitrosamine will be set to the 75% rather than the 50% tumor incidence level. We approximate that this will require administering nitrosamine for 28 weeks at the 400 μg daily dose. Administration of the two inhibitors will start 1 week prior to the beginning of dimethylnitrosamine administration. As in the synergism experiments, following termination of the administration schedule the animals will be maintained for an additional observational period of 11-12 months. Maintenance of the animals, autopsies, and histopathology will be as above.

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References:

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(2) Hoch-Ligeti, C., Argus, M. F., and Arcos, J. C. : "Combined Carcinogenic Effects of Dimethylnitrosamine and 3-Methylcholanthrene in the Rat". J. Natl. Cancer Inst. 40, 535 (1968).

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(4) Venkatesan, N., Argus, M. F., and Arcos, J. C. : "Mechanism of 3-Methylcholanthrene-induced Inhibition of Dimethylnitrosamine Demethylase in Rat Liver". Cancer Res. 30, 2556 (1970).

(5) Venkatesan, N., Arcos, J. C., and Argus, M. F. : " Amino Acid Induction and Carbohydrate Repression of Dimethylnitrosamine Demethylase in Rat Liver". Cancer Res. 30, 2563 (1970).

(6) Argus, M. F., Valle, R. T., Venkatesan, N., Buu-Hoi, N. P., and Arcos, J. C. : "Molecular-size-dependent effects of polynuclear hydrocarbons on mixed-function oxidases: possible action on cascade-coupled operons". Proc. 1st Europ. Biophys. Congr. EI/38, 187 (1971).

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(9) Wynder, E. L. : "Environmental Carcinogenesis and Cocarcinogenesis". Presented in Pharmacol. Symp. on "Mechanisms of Chemical Oncogenesis". 57th Federation Meet., Atlantic City, 1973, April 17.

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(12) Kunz, W., Schaude, G., and Thomas, C. : "Die Beeinflussung der Nitrosamincarcinogenese durch Phenobarbital und Halogenkohlenwasserstoffe". Z. Krebsforsch. 72, 291 (1969).

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Vol. I. Academic Press. 1961. p. 342

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Seamen's Memorial Research Laboratory at the U. S. Public Health Service Hospital has a total laboratory area of 4800 square feet, plus air-conditioned animal quarters of a total of 3000 square feet. The applicant has at his disposal approximately 1400 square feet of laboratory space, including a well-equipped cold room, approximately 2400 square feet of animal quarter space, and adequate office space. This is made available to the applicant through the Tulane Department of Medicine.

The major equipment in the applicant's laboratory space consists of the following: a Beckman DB spectrophotometer with recorder, a Shimadzu spectrophotometer with flow dichroism attachment, 4 Coleman spectrocolorimeters, a Photovolt fluorimeter, a Brice-Phoenix light-scattering photometer complete with ratio recorder, an Abbe refractometer, a Rudolph manual spectropolarimeter, 2 fourteen-manometer Warburg apparatuses, a "Yellow Springs" recording oxygen monitor, a high wattage ultrasonic generator, two analytical balances, several pH meters, a semi-automatic tensiometer, a "Spinco L" preparatory ultracentrifuge (including swinging bucket rotor and other accessories for polysome profiling), 3 "Servall" high-speed table centrifuges, 2 heavy-duty International model V centrifuges, a starch-block electrophoresis apparatus with circulating cooling system, a lyophilizing apparatus, 2 heating and cooling precision waterbaths, a Dubnoff waterbath incubator shaker; refrigerators, a deep freeze unit, an incubator. The use of a Microtech gas chromatograph and of an osmometer are available in the laboratory.

11. Additional facilities required:

Two project sites were given in the Summary Outline of this proposed project submitted to Dr. Hockett in our letter of May 25. The second site listed is very spacious and most modern animal quarters which our Department puts at our disposition in its newly acquired building (built 5 years ago by the Ethyl Corporation) which houses the Laboratory of Environmental Medicine. Although our animal facilities at the Seamen's Memorial Research Laboratory are adequate, in view of the potentially critical significance of this project for public health and for the cigarette industry, it would be preferable if the project could be carried out in these most up-to-date quarters. However, for us to be able to effectively occupy, in the newly acquired building, laboratory space commensurate with this project, we would need funds for installation costs and for adapting the space to our specific needs of bioassay studies. It is estimated that the costs to prepare space for this project in the Laboratory of Environmental Medicine would amount to $11,200.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

Curricula of three investigators appended.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

List of 10 publications see next page (reprints appended).

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13. Publications: (reprints enclosed).

(1) Argus, M. F., Sohal, R. S., Bryant, G. M., Hoch-Ligeti, C. and Arcos, J. C. : "Dose-Response and Ultrastructural Alterations in Dioxane Carcinogenesis. Influence of Methylcholanthrene on Acute Toxicity". Europ. J. Cancer 9, 237 (1973).

(2) Argus, M. F., Valle, R. T., Venkatesan, N., Buu-Hoi, N. P., and Arcos, J. C. : "Molecular-Size-Dependent Effects of Polynuclear Hydrocarbons on Mixed-Function Oxidases: Possible Action on Cascade--Coupled Operons". Proc. 1st Europ. Biophys. Congr. EI/38, 187 (1971).

(3) Argus, M. F., White, L. E., Bryant, G. M., Arcos, J. C. and Hoch-Ligeti, C. : "Molecular Specificity of the Tumorigenic Action of Ethionine: The Inactivity of S-Ethylcysteine. Action on Respiratory Parameters". Z. Krebsforsch. 75, 201 (1971).

(4) Arcos, J. C., Venkatesan, N., and Argus, M. F. : "Modification of the Flow Dichroism Spectrum of Rat Liver Nuclear DNA by In Vivo Alkylation with Hepatocarcinogenic Dialkylnitrosamines". Gann 62, 523 (1971).

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(8) Argus, M. F., Walder, J. A., Fabian, J. A., and Arcos, J. C. : " A Study of Soluble Protein and Sulfhydryl Levels in the Rat Liver During Rapid Normal and Premalignant Growth". Brit. J. Cancer 22, 330 (1968).

(9) Hoch-Ligeti, C., Argus, M. F., and Arcos, J. C. : "Combined Carcinogenic Effects of Dimethylnitrosamine and 3-Methylcholanthrene in the Rat". J. Natl. Cancer Inst. 40, 535 (1968).

(10) Arcos, J. C. and Simon, J. : " Effect of 4'-Substituents on the Carcinogenic Activity of 4-Aminoazobenzene Derivatives". Arzneim.-Forsch. (Drug Res.) 12, 270 (1962).

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Joseph C. Arcos, D. Sc.	R	R
Mary F. Argus, Ph. D.		
Joseph Simon, D. V. M., Ph. D.		
Technical		
Georgia M. Bryant, B. A., Technician	25%	$ 2,300
Laboratory Technician (to be recruited)	100%	$ 6,000
Laboratory Technician (to be recruited)	100%	$ 6,000
Animal caretaker (to be recruited)	100%	$ 4,450
Secretary (to be recruited)	40%	$ 2,700
	Sub-Total for A	25,450
B. Consumable supplies (by major categories)		
Animals		$ 1,200
Animal feed		1,600
Carcinogens & other Chemical Agents		2,400
Glassware		300
Animal Bedding & cleaning supplies for animal quarters		1,100
	Sub-Total for B	6,600
C. Other expenses (itemize)		
Installation & costs of adapting existing space for bioassay purposes (see 11. Additional facilities required)		$11,200
	Sub-Total for C	11,200
	Running Total of A + B + C	43,250
D. Permanent equipment (itemize)		
	Sub-Total for D	
E. Indirect costs (16% of A+B+C)	E	6,488
	Total request	49,738

15. Estimated future requirements: (see p. 12)

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	27,000	4,500	4,000	3,000	5,325	43,825
Year 3	28,600	3,300	9,000		6,135	47,035
Year 4	25,600	1,400	9,000		5,400	41,400

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Explanation of estimated requirements for future budget years:

1.) The salary category decreases in the 4th year, despite the annual increases, because we estimate that 1 technician will become unnecessary in the last 8 months of this project.

2.) The consumable supplies will be very low in the 4th year because the animal groups to be maintained are tapering off at that time.

3.) The category "Other Expenses" are histology costs. These are the lowest in the second year because the first experimental segment will begin to be processed for histopathology at the end of the second year. The major part of the histology cost requirements are distributed over the 3rd and 4th year.

4.) The category "Permanent Equipment" in the 2nd year is the cost to purchase replacement cages and racks. These will have to be purchased at the end of the 2nd year.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Faculty Research Award (exclusively salary grant)	American Cancer Society	127,887	1/1/72 - 12/31/76
Pharmacology of Repressible Mixed-Function Oxidases	NCI CA-13206	111,626	1/1/72 - 12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Pharmacology & Biochemistry of the Oncogen, Dioxane	NCI CA-15111	384,390	9/1/73 - 8/31/78
Control of Catabolism of Mitochondria in Oncogenesis	NCI	182,327	1/1/74 - 12/31/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph C. Arcos, D. Sc.

Signature Joseph Arcos Date 7/9/73

Telephone (504) 899-3441 331
Area Code Number Extension

Checks payable to

Tulane University

Mailing address for checks

Jesse B. Morgan, Comptroller

Tulane University, Station 20

New Orleans, La. 70118

Responsible officer of institution

Typed Name William G. Thurman, M. D.

Title Dean, Tulane Univ. School of Medicine

Signature William G. Thurman 7/9/73

Telephone (504) 588-5462
Area Code Number Extension

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R: REDACTED MATERIAL

CURRICULUM VITAE OF JOSEPH CHARLES ARCOS

PERSONAL DATA:

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Professional address:

Seamen's Memorial Research Laboratory
U. S. Public Health Service Hospital
210 State Street
New Orleans, Louisiana 70118

EDUCATIONAL BACKGROUND:

Undergraduate studies:

Secondary school: Gymnasium Baccalaureate degree (humanities - science). Nagykanizsa (Hungary), 1939.

1944-47. University "Bolyai" of Cluj (Roumania), Faculty of Sciences. "Licentiate in Chemistry" degree (minor: physics), "cum laude," 1947. Literature Thesis on "Chemistry and Physiological Effects of Vitamin D."

Graduate studies:

1947-50. Conservatoire National des Arts et Métiers, Paris, France. "Chemical Engineer" degree (major: dye chemistry; minor: industrial processes), "with felicitations," 1950. Thesis: "Synthèses de quelques nouveaux dérivés de la cystéine." (Synthesis of aminosulfonic, aminosulfinic and oxysulfonic acids related to cysteine and the separation of optical isomers). Subsequently, study of the competitive antagonism of these isomers with the isosteric aspartic acid in Lactobacillus helveticus and L. arabinosus.

1949-51. University of Paris, France. Faculty of Sciences. Preparation of a Doctoral Dissertation at the Collège de France. "Doctor in Science" degree in organic chemistry, "magna cum laude," 1951. Dissertation: "Recherches sur les diquinols mésodiphénylanthracéniques aminés. Étude sur la 'migration méso'." (Synthesis and photooxidation of hydroxy and amino derivatives of 9,10-diphenylanthracene. Elucidation of a new intramolecular rearrangement - leading to substituted anthrones). Literature Thesis (2nd Thesis): "La chimiothérapie de la lèpre."

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Post-graduate training:

1948: "Techniques in Physiology and Pharmacodynamics" (lectures and laboratory). Faculty of Medicine, University of Paris.

1951: Training periods in dye chemistry (syntheses and physicochemical studies), Francolor Co., Saint-Denis, France, and Imperial Chemical Industries, Manchester, England.

1952: "Oncology" (lectures and laboratory). Institut du Cancer "Gustave Roussy," University of Paris, Villejuif, France.

1969: American Chemical Society short course in "Molecular Orbital Theory." Houston, Texas.

POSITIONS:

1968- Professor of Medicine (Biochemistry). 1960-1968: Associate Professor of Medicine (Biochemistry), Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70112.

Principal research orientations: Alterations of mitochondrial electron transport parameters and membrane dynamics in the rat liver during hepatocarcinogenesis induced by amino azo dyes and nitrosamines, and in the rat heart during thiamine deficiency, following exercise, and following aortic constriction. Enzymology of extramitochondrial NADH oxidation. Phase-transitions in the mitochondrial membrane and change of stereochemistry of electron transport-related sulfhydryl groups during azo dye carcinogenesis; the use of dicarbonyl probes. Mechanism of apparent mitochondrial multiplication during feeding of noncarcinogenic azo dyes. Change of the electrophoretic pattern and sulfhydryl level in the soluble supernatant proteins of the rat liver in various situations of rapid growth. Structure-activity relationships of carcinogenic compounds in relation to their molecular geometry. Mechanism of dioxane carcinogenesis. Mechanism of protein denaturation by nitrosamines and related carcinogens; study of their hydrogen bonding ability. Effect of polynuclear hydrocarbons on microsomal azo dye and dimethylnitrosamine (DMN) metabolism in relation to their molecular geometry. Functional and kinetic characteristics of DMN-demethylase, the key enzyme of DMN carcinogenesis. Pre-repair geometry of nuclear DNA during DMN carcinogenesis. Genetic control of DMN-demethylase synthesis.

1957-1960: Assistant Research Professor, Cancer Research Laboratory, Department of Pharmaceutical Chemistry, University of Florida, In charge of the Section of Biophysical Chemistry.

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Research orientations: Alterations of the swelling of mitochondria in the rat liver during feeding amino azo dyes and other hepatic carcinogens, and in azo dye hepatomas; relationship with the onset of tumorigenesis. Non-linearity of the carcinogenic response. Effect of SH-reactants on mitochondrial swelling. Antagonism and synergism between carcinogenic azo dyes.

1953-1957: Project-Associate, McArdle Memorial Laboratory for Cancer Research, University of Wisconsin

Research orientations: Synthesis of derivatives of diphenylamine and of benzo(c)cinnoline, related to the mechanism of carcinogenesis by p-dimethylaminoazobenzene. Biological testing. The relation of molecular dimensions and of structural geometry to carcinogenic activity. Electrophoretic pattern and quantitative separation of fractions of the supernatant protein and of solubilized microsomal proteins of the rat liver; analysis of the amino acid composition, vitamin, nucleic acid and azo dye content of the fractions. Alterations of the swelling of rat liver microsomes during feeding of carcinogenic and noncarcinogenic azo dyes, and of azo dye hepatoma microsomes. Covalent binding of azo dyes to microsomes. Influence of various parameters on microsomal swelling.

1952-1953: Technical Assistant-Director. Jouan Company, Paris, France, manufacturers of laboratory apparatus. Planning and development of prototypes of a new closed circuit flame photometer, a semi-automatic polarograph, a differential galvanometer for the recording of hypo- and hyperthermy, a glossometer for the manufacturing of paper. Reorganization of the electronics division.

1952: Research Engineer. Technical Center of Graphic Industries, Paris, France. Physico-chemical studies in surface chemistry related to offset printing.

1940-1942: Development Assistant. Electronics Planning and Development Laboratory of the Tungsram (Orion) Corporation, Ujpest, Hungary.

PRINCIPAL SCIENTIFIC FINDINGS AND ACCOMPLISHMENTS:

---- Polynuclear aromatic hydrocarbons, phenobarbital, and a variety of other compounds are powerful inhibitors of dimethylnitrosamine demethylase activity, the key enzyme of carcinogenesis by this agent. This is due to repression of the de novo biosynthesis of the enzyme.

---- The ability of polynuclear aromatic hydrocarbons to induce the synthesis of certain microsomal mixed-function oxidases and to repress the synthesis of others depends on critical aspects of molecular size and shape, and the two effects show an exact mirror-image relationship.

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----- The concept that the genes coding for inducible and repressible mixed-function oxidases are located in separate but cascade-coupled operons (or groups of operons).

----- In vivo alkylation of nuclear DNA by nitrosamines leads to the appearance of "puffs" in the double helix ----- prior to excision and repair ----- due to disruption of the base-pairing pattern.

----- The large increase in the number of mitochondria per cell in the liver, during feeding of certain noncarcinogenic azo dyes ----- a phenomenon first noted around 1950 ----- is not due to de novo synthesis of the mitochondria, but to increase of their half-life.

----- The concept of "critical stage" in carcinogenesis, representing the point-of-no-return.

----- The critical stage in azo dye carcinogenesis correlates ----- in the target tissue ----- with specific alterations of: (a) the mitochondrial membrane, involving drastic temporary decrease of swelling-contraction and the temporary appearance of the phenomenon of respiratory release, and (b) the endoplasmic reticulum membrane as indicated by the sharp, temporary decrease of microsomal suspensions to show light-scattering changes attributed to swelling. Covalent binding of azo dyes in the two subcellular particles exclusively to membranes.

----- The sensitivity of the mitochondrial membrane to inducers of swelling decreases progressively in the target tissue of azo dye carcinogenesis and this is coincident with phase-transitions in the membrane.

----- The concept that the decrease of respiration observable in certain azo dye-induced hepatomas is due to electron leak in intermediate segment(s) of the electron transport chain, consequent to the biochemical lesions created by deletion of the molecular system of swelling-contraction.

----- The potent protein denaturing ability of some water-soluble carcinogens and the mechanism of this effect.

----- The carcinogenic activity of dioxane and certain aspects of its mechanism of action.

----- A p'-ethyl group is the strongest potentiator of activity of azo dye carcinogens (and probably aromatic amines).

----- Quantification of the relationship between molecular size of polynuclear aromatic hydrocarbons and their carcinogenic activity.

----- Cardiac hypertrophy of different etiologies all lead to decrease of mitochondrial ATP synthesis, but different patterns of alteration of the energy transducing system are involved.

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----- Alteration of extramitochondrial NADH oxidation and the existence of successive metabolic patterns in progressive thiamine deficiency.

----- In thiamine deficiency, decreased protein synthesis in heart tissue is mediated via decrease of the total ribosomal mass, rather than via the usual regulatory modality, i.e., shift in polysome/monosome ratio.

----- The mitochondrial membrane undergoes progressive changes during aging. The existence of host-transmissible factors regulating the dynamic state of the membrane.

----- The "meso migration," a new type of molecular rearrangement in polynuclear diquinols.

----- Writing of a multi-volume treatise on the "Chemical Induction of Cancer" (Academic Press). Three volumes completed.

GRANTS, AWARDS, FELLOWSHIPS:

1972-76. Faculty Research Award. American Cancer Society.

--- 1971. Principal Investigator. Research Grant-in-Aid, entitled "Inhibition of Azo Dye Carcinogenesis by Noncarcinogenic Analogs: Possible Mediation via Mitochondrial DNA Function," Cancer Association of Greater New Orleans, Inc.

1959- Principal Investigator. Research Grants C-4351 and C-5431, entitled "Finestructural Alterations in Cell Particles," from the National Cancer Institute of the United States Public Health Service.

1960-63. Co-investigator. Research Grant K-62-3, entitled "A Study of Sarcosomes in Normal and Dilated Heart," of the Hospital Division, United States Public Health Service.

--- 1960. Principal Investigator. Research Grant-in-Aid, entitled "Denaturation of Biological Macromolecules by Carcinogenic Agents," from the Greater New Orleans Cancer Association Inc. and the Cancer Society of Greater Baton Rouge.

--- 1950. Award of the Chamber of Dyers and Colorists of the City of Paris for majoring "with felicitations" in dye chemistry.

1948-50. Research Fellowship ("Stagiaire de Recherche"), National Center of Scientific Research (CNRS), Paris, France.

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MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

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Associate Editor: "Cancer Research," 1972-1976.
Editorial advisory board: "Cancer Research," 1968-71.
US National Cancer Plan panelist, Airlie House, December 1971.

LISTINGS IN PROFESSIONAL DIRECTORIES:

American Men of Science, Leaders in American Science.

LANGUAGES: French, German and Hungarian.

FOREIGN TRAVELS:

Resided, and travelled broadly in Central and Western Europe (Hungary, Roumania, Austria, Southern Germany, France, Belgium and England).

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PUBLICATIONS:
(in chronological order)

1. Etienne, A., and Arcos, J. C., "Recherches sur les diquinols mésoamino-diphénylanthracéniques. I. Mésodiphénylanthracènes aminés en para sur les phényles." Bull. Soc. Chim. France, 18, 684-689 (1951).

2. Etienne, A. and Arcos, J. C., "Recherches sur les diquinols mésoamino-diphénylanthracéniques. II. Migration d'aryles des diquinols bis-p-dialcoylaminés sur les phényles." Bull. Soc. Chim. France, 18, 690-693 (1951).

3. Arcos, J. C., "Recherches sur les diquinols mésodiphénylanthracéniques aminés. Étude sur la 'migration méso'." Monograph Soc. Édition Enseignement Supérieur, Paris, 1951 (Printed Doctoral Dissertation).

4. Arcos, J. C. and Miller, J. A., "Synthesis of 2-Amino-5-dimethylaminodiphenylamine and Other Derivatives of 3,4-Dinitrodimethylaniline." J. Am. Chem. Soc., 77, 3128-3129 (1955).

5. Arcos, J. C., Arcos, M., Buu-Hoi, N. P. and Lacassagne, A., "Indices de Valence Libre, Adsorbabilités et Activité Cancerogène des Hydrocarbures Polycycliques." Comptes Rendus Acad. Sci., 241, 836-839 (1955).

6. Arcos, J. C. and Arcos, M., "Activité Cancerogène et Relation Structurale Entre le Graphite et les Hydrocarbures Polycycliques Condensés." Comptes Rendus Acad. Sci., 241, 777-779 (1955).

7. Arcos, J. C. and Arcos, M., "Size and Carcinogenic Activity of the Polycyclic Hydrocarbons." Naturwissenschaften, 42, 608 (1955).

8. Arcos, J. C. and Arcos, M., "Inclusion Compounds and Carcinogenesis: A Stereochemical Model." Naturwissenschaften, 42, 651-652 (1955).

9. Arcos, J. C. and Arcos, M., "Stereochemical Factors in Carcinogenesis." Bull. Soc. Chim. Belges, 65, 5-16 (1956).

10. Arcos, J. C., Arcos M. and Miller, J. A., "Synthesis of Nitro and Amino Derivatives of Benzo(c)cinnoline." J. Org. Chem., 21, 651-654 (1956).

11. Arcos, J. C. and Arcos, M., "Géométrie moleculaire et carcinogénèse chimique." Rev. Fermentations (Belgium), 11, 127-134 (1956).

12. Arcos, M. and Arcos, J. C., "Sur le Rôle Possible du Graphite dans la Biogénèse." Rev. Gen. Sci. et Bull. Assoc. Franc. Avancement des Sciences, 63, 89-100 (1956).

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13. Arcos, J. C. and Arcos, M., "An Evidence for Change in Microsomal Fine-structure During Chemical Carcinogenesis." Naturwissenschaften, 44, 331-332 (1957).

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14. Arcos, J. C., "Studies on the Swelling and Solubilization of Rat Liver Microsomes." Naturwissenschaften, 44, 332 (1957).

15. Arcos, M. and Arcos, J. C., "Sur un mécanisme de l'augmentation de l'activité de composés cancerogènes par certains substituants fluorés et alcoylés." Comptes Rendus Acad. Sci., 245, 1092-1094 (1957).

16. Arcos, J. C. and Arcos, M., "Finestructural Alterations in Cell Particles During Chemical Carcinogenesis. I. Influence of the Feeding of Aminoazo Dyes on the Swelling and Solubilization of Rat Liver Microsomes." Biochim. Biophys. Acta, 28, 9-20 (1958).

17. Lacassagne, A., Arcos, J. C., Buu-Hoi, N. P. and Zajdela, F., "Quelques expériences préliminaires sur le mécanisme de la cancérisation par matières plastiques." Bull. Cancer, 44, 545-551 (1957).

18. Wirtz, G. H. and Arcos, J. C., "Studies on the Dye-Binding Fraction of Soluble Liver Proteins from Rats Fed Aminoazo Dyes." Experientia, 14, 177-178 (1958).

19. Arcos, M. and Arcos, J. C., "A Correlation of Constitution and Carcinogenic Activity. Part I. A Study of the Essential Molecular Parameters." Arzneimittelforschung (Drug Research) 8, 486-489 (1958).

20. Arcos, J. C. and Arcos, M., "A Correlation of Constitution and Carcinogenic Activity. Part II A. Interactions Involving Covalent Bonds of Carcinogens with Proteins in the Cell. An Attempt of Evaluation." Arzneimittelforschung (Drug Research) 8, 602-605 (1958).

21. Arcos, M. and Arcos, J. C., "A Correlation of Constitution and Carcinogenic Activity. Part II B. Interactions Involving Secondary Valence Forces of Carcinogens with Proteins in the Cell. New Developments." Arzneimittelforschung (Drug Research) 8, 643-647 (1958).

22. Arcos, J. C., Griffith, G. W. and Cunningham, R. W., "Studies on the Swelling of Rat Liver Mitochondria in Relation to Tumor Incidence During Feeding of Aminoazo Dyes." Experientia, 15, 316-317 (1959).

23. Arcos, J. C., Griffith, G. W. and Cunningham, R. W., "Finestructural Alterations in Cell Particles During Chemical Carcinogenesis. II. Further Evidence for Their Involvement in the Mechanism of Carcinogenesis. The Swelling of Rat Liver Mitochondria During Feeding of Aminoazo Dyes." J. Biophys.-Biochem. Cytol., 7, 49-60 (1960).

24. Arcos, J. C., Conney, A. H., and Buu-Hoi, N. P., "Induction of Microsomal Enzyme Synthesis by Polycyclic Aromatic Hydrocarbons of Different Molecular Sizes." J. Biol. Chem., 236, 1291-1296 (1961).

25. Arcos, J. C., "Alterations of Microsomal and Mitochondrial Sites of Metabolic Control During Chemical Carcinogenesis." Bull. Tulane Med. Fac., 20, 133-150 (1961).

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26. Gosch, H.H., and Arcos, J.C., "Transmission of Swelling Characteristics by Mitochondria of Regenerating Liver". Nature, 190, 272-273 (1961).

27. Arcos, J.C., Gosch, H.H., and Zickafoose, D., "Finestructural Alterations in Cell Particles During Chemical Carcinogenesis. III. Selective Action of Chemical Carcinogens Other Than 3'-Methyl-4-Dimethylaminoazobenzene. Effect of Stimulated Liver Growth." J. Biophys. Biochem. Cytol., 10, 23-36 (1961).

28. Arcos, J.C., and Griffith, G.W., "Ratio Dependent Synergism in Azo Dye Carcinogenesis". Brit. J. Cancer, 15, 291-298 (1961).

229. Arcos, J.C., and Simon, J., "Effect of 4'-Substituents on the Carcinogenic Activity of 4-Aminoazobenzene Derivatives". Arzneimittelforschung (Drug Research), 12, 270-275 (1962).

30. Gosch, H.H., Arcos, J.C., and Argus, M.F., "Kinetics of Mitochondrial Swelling: Evidence for Specific Receptor Sites for Inducer Compounds." Nature, 195, 1179-1181 (1962).

31. Argus, M.F., Arcos, J.C., and Walder, J.A., "An Evidence for Decrease of Energy Production in Thiamine Deficient Failing Rat Heart." Experientia 28, 519-521 (1962).

32. Argus, M.F., and Arcos, J.C., "Failure of Monochloroacetone to Influence 3,4-Benzpyrene Mouse Skin Tumorigenesis". Experientia, 19; 588-589 (1963).

33. Arcos, J.C., Walder, J.A., and Argus, M.F., "Inhibition and Reversal of Swelling of Rat Heart Sarcosomes by Reduced Diphosphopyridine Nucleotide". Nature, 201, 398-400 (1964).

34. Argus, M.F., Arcos, J.C., Alam, A., and Mathison, J.H., "A Viscometric Study of Hydrogen-Bonding Properties of Carcinogenic Nitrosamines and Related Compounds". J. Medicinal Chem., 7, 460-465 (1964).

35. Arcos, J.C., and Argus, M.F., "A Reconsideration of the Isolation in Presence of EDTA and the Swelling-Contraction Characteristics of Rat Heart Sarcosomes. Metabolic Topography of the Sarcosomal Contractile Protein", Biochemistry, 3, 2028-2040 (1964).

36. Arcos, J.C., Argus, M.F., Sardesai, V.M., and Stacey, R.E., "Effect of Thiamine Deficiency on the Swelling, Electron Transport and Oxidative Phosphorylation of Rat Heart Sarcosomes", Biochemistry, 3, 2041-2045 (1964).

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37. Argus, M.F., Arcos, J.C., Sardesai, V.M., and Overby, J.L., "Oxidative Rates and Phosphorylation in Sarcosomes From Experimentally-Induced Failing Rat Heart", Proc. Soc. Exptl. Biol. Med., 117, 380-383 (1964).

38. Arcos, J.C., Wirtz, G.H., and Arcos, M., "Sur la possibilité de production de tumeurs malignes par des anticorps organo-spécifiques: une méthode de vérification de la théorie d'élimination d'enzyme dans la carcinogénèse chimique expérimentale", Compt. Rend. Acad. Sci., 260, 2371-2373 (1965).

39. Argus, M.F., Arcos, J.C. and Hoch-Ligeti, C., "Studies on the Carcinogenic Activity of Protein Denaturing Agents: Hepatocarcinogenicity of Dioxane". J. Natl. Cancer Inst., 35, 949-958 (1965).

40. Argus, M.F., Arcos, J.C., Mathison, J.H., Alam, A. and Bemis, J.A., "Studies on the Denaturation of Biological Macromolecules by Chemical Carcinogens. I. Aggregation, Viscosity and Optical Rotation Changes Produced in Ovalbumin by Nitrosamines and Other Water-Soluble Carcinogens". Arzneimittelforschung (Drug Research), 16, 740-746 (1966).

41. Argus, M.F., Arcos, J.C., Mathison, J.H. and Alam, A., "Studies on the Denaturation of Biological Macromolecules by Chemical Carcinogens. II. The Reactivity of Sulfhydryl Groups of Ovalbumin. The Sulfhydryl-Disulfide Interchange Mechanism of Aggregation Induced by Nitrosamines and Other Water-Soluble Carcinogens." Arzneimittelforschung (Drug Research), 16, 1083-1088 (1966).

42. Bemis, J.A., Argus, M.F., and Arcos, J.C., "Studies on the Denaturation of Biological Macromolecules by Chemical Carcinogens. III. Optical Rotatory Dispersion and Light-Scattering Changes of Ovalbumin During Denaturation and Aggregation by Water-Soluble Carcinogens". Biochim. Biophys. Acta, 126, 274-285 (1966).

43. Arcos, J.C., Stacey, R.E., Mathison, J.B., and Argus, M.F., "Kinetic Parameters of Mitochondrial Swelling. The Effect of Animal Age. Tissue Distribution of the Mitochondrial "Contractile Protein". Exper. Cell Res., 48, 448-460 (1967).

44. Arcos, J.C., Sohal, R.S., Sun, S.C., Argus, M.F., and Burch, G.E., "Changes in Ultrastructure and Respiratory Control in Mitochondria of Rat Heart Hypertrophied by Exercise". Exper. Mol. Path, 8, 49-65 (1968).

45. Hoch-Ligeti, C., Argus, M.F., and Arcos, J.C., "Combined Carcinogenic Effects of Dimethylnitrosamine and 3-Methylcholanthrene in the Rat". J. Natl. Cancer Inst., 40, 535-549 (1968).

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46. Bemis, J.A., Bryant, G.M., Arcos, J.C., and Argus, M.F., "Swelling and Contraction of Mitochondrial Particles: A Re-examination of the Existence of a Contractile Protein Extractable with 0.6 M-Potassium Chloride". J. Mol. Biol., 33, 299-307 (1968).

47. Argus, M.F., Walder, J.A., Fabian, J.A., and Arcos, J.C., "A Study of Soluble Protein and Sulfhydryl Levels in the Rat Liver during Rapid Normal and Premalignant Growth". Brit. J. Cancer, 22, 330-341 (1968).

48. Venkatesan, N., Arcos, J.C., and Argus, M.F.: "Differential Effect of Polycyclic Hydrocarbons on the Demethylation of the Carcinogen Dimethylnitrosamine by Rat Tissues". Life Sciences, 7, (Part I), 1111-1119 (1968).

49. Arcos, J.C., Mathison, J.B., Tison, M.J., and Mouledoux, A.M., "Effect of Feeding Amino Azo Dyes on Mitochondrial Swelling and Contraction. Kinetic Evidence for Deletion of Membrane Regulatory Sites." Cancer Research, 29, 1288-1297 (1969).

50. Arcos, J.C., Tison, M.J., Gosch, H.H., and Fabian, J.A. "Sequential Alterations in Mitochondrial Inner and Outer Membrane Electron Transport, and in Respiratory Control during Feeding Amino Azo Dyes; Stability of Phosphorylation. Correlation with Swelling-Contraction Changes and Tumorigenesis Threshold". Cancer Research, 29, 1298-1306 (1969).

51. Arcos, J.C., Argus, M.F., and Mathison, J.B., "Hepatic Carcinogenesis Threshold and Biphasic Mitochondrial Swelling Response in the Guinea Pig During Diethylnitrosamine Administration". Experientia, 25, 296-298 (1969).

52. Buu-Hoi, N.P., Saint-Ruf, G., and Arcos, J.C., "Sur quelques hétérocycles dérivés d'orthodiamines pyridiques, pyrimidiques et indazoliques". Bull. Soc. Chim. France 838-842 (1969).

53. Gosch, H.H., Arcos, J.C., and Argus, M.F., "On the Unimpairable Resistance of the Guinea Pig to Dietary Amino Azo Dye Hepatocarcinogenesis". Z. Krebsforsch., 73, 215-217 (1970).

54. Hoch-Ligeti, C., Argus, M.F., and Arcos, J.C., "Induction by Dioxane of Carcinomas in the Nasal Cavity of Rats". Brit. J. Cancer, 24, 164-167 (1970).

55. Wu, B.C., Argus, M.F., and Arcos, J.C., "Differential Decrease of "Shuttle" Enzymes of Extra-mitochondrial $NADH_2$ Oxidation of Heart Muscle during Progressive Thiamine Deficiency". Proc. Soc. Exptl. Biol. Med., 133, 808-812 (1970).

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56. Venkatesan, N., Argus, M.F., and Arcos, J.C., "Mechanism of Methylcholanthrene-Induced Inhibition of Dimethylnitrosamine-Demethylase in Rat Liver". Cancer Res., 30, 2556-2562 (1970).

57. Venkatesan, N., Arcos, J.C., and Argus, M.F., "Amino Acid Induction and Carbohydrate Repression of Dimethylnitrosamine-Demethylase in Rat Liver". Cancer Res., 30, 2563-2567 (1970).

58. Arcos, J.C., "Ultrastructural Alteration of the Electron Transport Chain Involving Electron Leak: Possible Basis of "Respiratory Impairment" in Certain Tumors." J. Theoret. Biol. 30, 533-543 (1971).

59. Argus, M.F., White, L.E., Bryant, G.M., Arcos, J.C., and Hoch-Ligeti, C., "Molecular Specificity of the Tumorigenic Action of Ethionine; the Inactivity of S-Ethylcysteine. Action on Respiratory Parameters". Z. Krebsforsch., 75, 201-208 (1971).

60. Wu, B.C., Arcos, J.C., and Argus, M.F., "Polysome Profile and Change in Total Ribosomal Mass in Rat Heart During Progressive Thiamine Deficiency". Life Sciences, 10 (Part II), 541-551 (1971).

61. Venkatesan, N., Arcos, J.C., and Argus, M.F., "Induction and Repression of Microsomal Drug-Metabolizing Enzymes by Polycyclic Hydrocarbons and Phenobarbital: Theoretical Models". J. Theoret. Biol., 33, 517-537 (1971).

62. Argus, M.F., Valle, R.T., Venkatesan, N., Buu-Hoi, N.P., and Arcos, J.C., "Molecular Size-Dependent Effects of Polynuclear Hydrocarbons on Mixed-Function Oxidases: Possible Action on Cascade-Coupled Operons". Proceedings First Europ. Biophys. Congr. (Vienna) 1 (EI/38), 187-192 (1971).

63. Wu, B.C., Valle, R.T., White, L.E., Sohal, R.S., Arcos, J.C., Argus, M.F., and Burch, G.E., "Alterations in the Energy Transducing System and Mitochondrial Ultrastructure of Rat Heart During Progressive Thiamine Deficiency". Virchows Arch. Abt. B Zellpath. 9, 97-114 (1971).

64. Arcos, J.C., Venkatesan, N., and Argus, M.F., "Modification of the Flow Dichroism Spectrum of Rat Liver Nuclear DNA by In Vivo Alkylation with Heptocarcinogenic Dialkylnitrosamines". Gann (Japanese J. Cancer Res.) 62, 523-533 (1971).

65. Buu-Hoi, N.P., Saint-Ruf, G., and Arcos, J.C., "Synthèse, Structure et Propriétés de Dérivés Nouveaux de Triaza-, Tetraaza- et Pentaaza--benz [a] anthracènes." Bull. Soc. Chim. France 2028-2032 (1972).

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66. Argus, M. F., Sohal, R. S., Bryant, G. M., Hoch-Ligeti, C., and Arcos, J. C., "Dose-response and Ultrastructural Alterations in Dioxane Carcinogenesis. Influence of Methylcholanthrene on Acute Toxicity." Europ. J. Cancer, 9, 237-246 (1973).

67. Wu, B. C., Arcos, J. C., and Argus, M. F.: "Control of Catabolism of Mitochondria in Oncogenesis. I. Increase of Hepatic Mitochondrial Population During Feeding Inactive or Marginally Active Amino Azo Dyes: Absence of de Novo Biosynthesis". Submitted for publication.

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Monographs and Contributions to Review Series & Symposia.

1. Arcos, J. C., and Arcos, M.: "Molecular Geometry and Mechanisms of Action of Chemical Carcinogens." In "Progress in Drug Research," Jucker, E., Editor, Birkhäuser, Basel, Vol. 4, 1962, pp. 407-581.

2. Arcos, J. C.: "Carcinogenic Substances." In "Encyclopedia of Biochemistry," Williams, R. J., and Lansford, E. M., Eds., Reinhold, New York, 1967, pp. 192-196.

3. Arcos, J. C., Argus, M. F. and Wolf, G.: "Chemical Induction of Cancer — Structural Bases and Biological Mechanisms" Vol. I. (This volume contains Part I. Molecular architecture and the physical bases of molecular forces; and Part II. The nature of tumors. Concepts and techniques of testing chemical agents for carcinogenic activity.) Academic Press, New York, 1968, 491 pages.

4. Arcos, J. C., and Argus, M. F.: "Molecular Geometry and Carcinogenic Activity of Aromatic Compounds. New Perspectives." In "Advances in Cancer Research," Academic Press, New York, Vol. 11, 1968, pp. 305-471.

5. Arcos, J. C.: "French Research on Chemical Carcinogenesis in the Last Quarter Century with Special Reference to the Contributions of A. Lacassagne and N. P. Buu-Hoi." Proceedings of "World Symposium on Model Studies in Chemical Carcinogenesis," Baltimore, Oct. 31-Nov. 3, 1972. Marcel Dekker publ., in press.

6. Arcos, J. C., and Argus, M. F.: "Chemical Induction of Cancer ----- Structural Bases and Biological Mechanisms." Vol. II A. (This volume contains the segment on Polynuclear Compounds of Part III. "Structure-Activity Relationships of Chemical Carcinogens. Effect of Chemical Reactivity, Molecular Geometry, and Metabolism on Carcinogenic Activity"). Academic Press, New York, in press, scheduled to appear 1973.

7. Arcos, J. C., and Argus, M. F.; "Chemical Induction of Cancer ----- Structural Bases and Biological Mechanisms." Vol. II B. (This volume contains the segment on Aromatic Amines and Nitro Compounds, Arylazo Compounds, and N-Hydroxy Compounds, of Part III. "Structure-Activity Relationships of Chemical Carcinogens. Effect of Chemical Reactivity, Molecular Geometry, and Metabolism on Carcinogenic Activity"). Academic Press, New York, in press, scheduled to appear 1973.

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Abstracts and Science - Related Notes

1. Arcos, J. C., and Arcos, M.: "Stereochemical Factors in Carcinogenesis." Vol. of Abstracts, p. 129, 3rd Internat. Congress of Biochemistry, Brussels, 1955.

2. Miller, J. A., Arcos, J. C. and Miller, E. C.: "The Requirement of a Free 2-Position for the Carcinogenicity of 4-Dimethylaminoazobenzene." Proc. Am. Assoc. Cancer Res., 2, 32 (1955).

3. Arcos, J. C. and Griffith, G. W.: "Finestructural Alterations in Cell Particles During Chemical Carcinogenesis." Federation Proc., 18, 182 (1959).

4. Arcos, J. C., Zickafoose, D. and Warren, R. D.: "Selective Alteration of Liver Mitochondria Caused by Hepatic Carcinogens." Federation Proc., 19, 55 (1960).

5. Arcos, J. C. and Griffith, G. W.: "Antagonism and Synergism in Hepatic Carcinogenesis with Reference to Mixed Crystal Formation." Proc. Am. Assoc. Cancer Research, 3, 92 (1960).

6. Arcos, J. C., and Gosch, H. H.: "Swelling of Mitochondria from Hepatoma and During Induced Liver Growth in the Rat." Proc. Am. Assoc. Cancer Research, 3, 206 (1961).

7. Argus, M. F., and Arcos, J. C.: "Mechanism of Ovalbumin Denaturation by Nitrosamines and Other Carcinogens." Federation Proc. 23, 523 (1964).

8. Arcos, J. C., and Argus, M. F.: "Metabolic Topography of the "Contractile Protein" in Heart Sarcosomes." Sixth Internat. Congress of Biochemistry, New York. Research Reports, 8, 643 (1964).

9. Arcos, J. C." "Regulatory Pathways of Mitochondrial Morphology and Oxidative Phosphorylation: Alterations During Azo Dye Carcinogenesis." Ninth International Cancer Congress, Tokyo, 1966. Volume of Abstracts, p. 196, abstract SO318.

10. Bemis, J. A., Arcos, J. C., Argus, M. F. and Bryant, G. M.: "The Sarcosomal Contractile Protein (CP): Superprecipitation Properties and Molecular Characteristics." Federation Proc., 26, 827 (1967).

11. Argus, M. F., and Arcos, J. C.: "Industry's Image in Schools" in 'Letters' Chem. & Eng. News 45 (No. 3), 6 (1967).

12. Argus, M. F., and Arcos, J. C.: "Industry and the Ivory Tower" in 'Letters.' Chem. & Eng. New. 45 (No. 7), 6 (1967).

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13. Venkatesan, N., Arcos, J.C., and Argus, M.F.: "Regulatory Mechanisms in the Demethylation of Dimethylnitrosamine (DMN)." Tenth International Cancer Congress, Houston, 1970. Volume of Abstracts p. 11-12, abstract No. 15.

14. Arcos, J.C., Bryant, G.M., and Argus, M.F.: "Stereoselective Uncoupling of Oxidative Phosphorylation by Aliphatic Dicarbonyls: Probes in Azo Dye Carcinogenesis." Tenth International Cancer Congress, Houston, 1970. Volume of Abstracts, p. 48-49, abstract No. 74.

15. Arcos, J.C.: "Differential Effect of Diacetyl on Oxidative Phosphorylation and Swelling of Rat Liver Mitochondria in the Course of Azo Dye Carcinogenesis." Ann. Meet. Southwest Section Am. Assoc. Cancer Res., New Orleans, 1970. Vol. Abstracts.

16. Arcos, J.C., Venkatesan, N., and Argus, M.F.: "Flow Dichroism of Rat Liver Nuclear DNA Alkylated in Vivo by Nitrosamines." 15th Ann. Meet. Biophys. Soc., New Orleans, 1971. Abstracts, abstr. # ThAM-L 10, p. 301a.

17. Arcos, J.C.: "Comments to Hadler's 'Unitary Hypothesis for Carcinogenesis'". Bioenergetics Bulletin 3 [4], 24-26 (1971).

18. Arcos, J.C.: "In Memory of Nguyen Phuc Buu-Hoi: 1915-1972." (an obituary). Cancer Res., 32, 2856A - 2856D (1972).

19. Arcos, J.C., Argus, M.F., and Buu-Hoi, N.P.: "Repression of Dimethylnitrosamine (DMN) Demethylase by Polynuclear Hydrocarbons and Other Compounds." Federation Proc. 32, 702 Abs (1973).

20. Arcos, J.C., Wu, B.C., and Argus, M.F.: "Increase of Hepatic Mitochondrial Population During Feeding Noncarcinogenic Azo Dyes: Apparent Absence of de Novo Biosynthesis." Proc. Am. Assoc. Cancer Res. 14, 45 (1973).

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CURRICULUM VITAE OF MARY FRANCES ARGUS

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Education; REDACTED

Secondary - St. Joseph High School, Ironton, Ohio (R) Latin Scientific Course; Valedictorian; Recipient of four year college tuition scholarship.

Undergraduate - B. S. (magna cum laude) (Major-Chemistry, Minors-Mathematics, English; Certificate to teach Science, Mathematics and English in Secondary Schools in the State of Ohio) - (R) College of Mount Saint Joseph-on-the-Ohio, Cincinnati, Ohio.

Graduate - M. S. (Major- Organic Chemistry, Minor-Physical Chemistry, Thesis - "Sulfur Derivatives of Fluorene") - June 1947 - University of Cincinnati, Cincinnati, Ohio.

Ph. D. (Major-Oncology, Minor-Pharmacology, Thesis - "New Compounds for Cancer Research. The Synthesis and Metabolic Study of Isotopic Derivatives of Fluorene.") - February 1952 - University of Florida, Gainesville, Florida.

Specialized - Oak Ridge Institute of Nuclear Studies: Advanced Course for Biochemists - November; 1952; Course in Advanced Instrumentation - July, 1954. American Chemical Society Short Course in "Molecular Orbital Theory", Houston, 1969. The Jackson Laboratory "Short Course in Mammalian Genetics", Bar Harbor, Maine, 1969.

Professional Experience:

September 1945 - June 1947 - Assistant Instructor of General and Organic Chemistry, University of Cincinnati , Cincinnati, Ohio.

September 1947 - June 1949 - Instructor of General, Organic and Biochemistry, Marygrove College, Detroit, Michigan.

February 1952 - June 1956 - Assistant Research Professor in charge of Biochemistry Section, Cancer Research Laboratory, University of Florida, Gainesville, Florida.

July 1956 - October 1960 - Associate Research Professor in charge of Biochemistry Section, Cancer Research Laboratory (affiliated with Dept. of Pharmaceutical Chemistry, College of Pharmacy from July, 1959), University of Florida, Gainesville, Florida.

November, 1960 - May, 1968 - Associate Professor of Medicine (Biochemistry), Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana, and Seamen's Memorial Research Laboratory, U.S. Public Health Service Hospital, New Orleans, La.

May, 1968 - - Professor of Medicine (Biochemistry), Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana, and Seamen's Memorial Research Laboratory, U.S. Public Health Service Hospital, New Orleans, La.

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Professional Organizations:

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Listed in: American Men of Science, Who's Who of American Women, Leaders in American Science.

Research Grants:

1. Atomic Energy Commission Contract AT-(40-1)-2097
   "Studies of Radiation and Chemical Carcinogenesis"

2. U.S. Public Health Service Research Grant C-1356
   "Localization of Radioactive Compounds in Tumors"

3. U.S. Public Health Service Research Grant CY-4939
   "Mechanism of Nitrosamine Carcinogenesis"

4. Greater New Orleans Cancer Association
   "Denaturation of Biological Macromolecules by Carcinogenic Agents"

5. U.S. Public Health Service Research Grant C-5793
   "Mechanism of Nitrosamine Carcinogenesis"

6. Division of Hospitals, U.S. Public Health Service, Grant K62-3
   "Study of Sarcosomes in Normal & Dilated Heart"

7. Greater New Orleans Cancer Association
   "Mechanism of Gene-Level Inhibition of Carcinogenesis"

8. U.S. Public Health Service Research Grant CA-13206
   "Pharmacology of Repressible Mixed-Function Oxidases"

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EDUCATION:

Cornell University	1936-1940	B.S.	Dairy Manufacturing
Texas A. and M. College	1941-1942	M.S.	Dairy Manufacturing
Kansas State College	1943-1946	D.V.M.	Veterinary Medicine
University of Wisconsin	1948-1951	Ph.D.	Veterinary Medicine Pathology and Bacteriology

1946-1948 Veterinary Practice and Regulatory Work

1948-1958 Instructor and Assistant Professor, Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin

September 1958 to May 1960 Research Associate Professor, Cancer Research Laboratory, University of Florida, Gainesville, Florida

May 1960 to Present Professor Pathology, Hygiene and Veterinary Research College of Veterinary Medicine, University of Illinois, Urbana, Illinois

RESEARCH: Projects pertaining to bovine mastitis, reproductive diseases of dairy cattle and mycotic agents of importance to cattle, including microbiological, pathological, therapeutic and field aspects of these problems; experimental pathology pertaining to carcinogenesis of various agents. Supervision of graduate students, technicians, farm laborers, animal caretakers and part-time workers. Approximately 90 scientific papers published.

TEACHING: Courses in Veterinary Pathology for undergraduate and graduate students, course in oncology for graduate students, charge of clinical-pathologic conference for 3rd and 4th year students.

CONSULTATION: (a) Members of the Animal Disease Diagnostic Laboratory of the State of Wisconsin, local veterinary practitioners, dairy field men and farmers. Much of the work consisted of gross and histopathology as well as bacteriology.

(b) Projects in the Departments of Agronomy, Genetics, Bacteriology and Dairy Industry at the University of Wisconsin.

(c) Departments of Veterinary Science and Bacteriology at the University of Florida.

(d) Departments of Dairy Science and Animal Nutrition, University of Illinois.

VETERINARY EXPERIENCE: Approximately two years in practice prior to entrance into the graduate school of the University of Wisconsin in 1948.

VETERINARY LICENSING: Wisconsin, Montana, Kansas and Missouri

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R: REDACTED MATERIAL

PROFESSIONAL SOCIETY MEMBERSHIPS:

HONORARY SOCIETY MEMBERSHIPS:

MISCELLANEOUS AFFILIATIONS

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#920 - BENEDICT

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 19, 1973

Grant Application No. 920

To: The committee comprising Drs. Andervont, Gardner, and Huebner

Subject: William F. Benedict, M.D., Childrens Hospital of Los Angeles
New application No. 920
"Chromosomal Changes in Explanted Human Cancer Cells and Hamster Trachea Organ Cultures."

History

Currently Dr. Benedict has a "service contract" from CTR, $10,000, July 1, 1973 through June 30, 1974, "Relationship between Cell Transformation, Chromosomal Changes and Carcinogenesis".

Application No. 920 requests approximately $25,700, plus one additional year. The budget appearing on page 5 is being revised by the applicant as an incorrect indirect cost rate was used.

Documents Submitted

Attached is application (undated, 7 pages) received July 13, 1973. Also attached are C.V.'s of Drs. Benedict, Baker, Landing and Isaacs.

Reprints of publications listed as numbers 3, 5, 6, 8 and 15 in the C.V. of Dr. Benedict have been provided; they will be forwarded to you on request.

Comment

Dr. Kreisher, who is in close touch with Dr. Benedict, has volunteered to comment on this proposal. This comment will follow in due course.

F.W.N.

FWN:wg
Encls.
cc: Dr. Kreisher

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Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

#920

JUL 13 1973

1. Principal Investigator (give title and degrees)

William F. Benedict, M.D.
Assistant Professor of Pediatrics,
USC School of Medicine

2. Institution and Address:

Divisions of Hematology-Oncology and Medical Genetics,
Childrens Hospital of Los Angeles
4650 Sunset Boulevard,
Los Angeles, California. 90054.

3. Department(s) where research will be done or collaboration provided

Divisions of Hematology-Oncology and Medical Genetics,
Childrens Hospital of Los Angeles

4. Short title of study:

a. Relationship of chromosomal changes to human malignancy in a human-mouse/human-hamster model system.

b. Chromosome changes, metaplasia and carcinogenesis in hamster tracheal organ culture.

5. Proposed starting Date:

December 1, 1973

6. Estimated time to complete:

Two years.

7. Brief description of specific research aims:

A. Firstly we shall attempt to study the importance of specific human chromosomes in malignancy by the use of a human-mouse or human-hamster model system recently developed. Both near diploid human carcinomas and sarcomas will be injected into mice or hamsters which have been previously treated with anti-thymocyte serum. Chromosomal analysis will be made of the tumor cells to

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identify similar increases or decreases in particular chromosomes using a trypsin-Giemsa banding technique which enables each human chromsome to be easily identified (10). Revertant cells will be isolated which have decreased tumor producing potential and their chromosomal pattern will also be studied. Finally, all tumor lines established at the Childrens Hospital of Los Angeles over the next two years will be similarly studied in the human-hamster and/or human-mouse system.

B. Secondly, we shall study hamster tracheal epithelial tissue in organ culture. Mary Baker from Dr. Michael Sporn's laboratory at the Lung Cancer Unit of the NCI will be joining our group in September. She has been one of the pioneers in developing this system and now hamster tracheal tissue can be maintained for extended periods.

We shall use this system to study the evolution of epithelial metaplasia and hopefully carcinoma in situ by testing these cultures with various carcinogens, particularly polycyclic hydrocarbons: Chromosomal analysis will be done on the early changes toward malignancy to see if the same "expressor" and "suppressor" chromosomes are involved as has previously been reported in chemically induced transformation of hamster embryo cultures (3). These studies are a matural retention of other CTR projects being done in collaboration with Dr. R. Kouri and Dr. C. Whitmine at Microbiological Associates on endotracheal produced polycyclic hydrocarbon carcinogenesis in the mouse.

8. Background

A. The importance of the balance of specific hamster "expressor" and "suppressor" chromosomes in virally (1) or dimethylnitrosamine (2) produced malignant transofmration has recently been reported. We have found the same "expressor" and "suppressor" chromosome involvement in 1-β-D-arabinofuranosylcytosine (ara-C) transformation as previously had been found with dimethylnitrosamine (3). Thus it will be interesting to see whether or not a similar chromosomal relationship will be found with other chemical carcinogens such as polycyclic hydrocarbons. We have also recently begun studying chromosomal changes in methylcholantrene transformed C_3H mouse cells. Preliminary data suggests the presence of "suppressor" chromosomes in the mouse system as well. Threfore, since there is evidence of chromosomes which may influence the expression of malignancy in two mammalian systems, it is important to see if a

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similar relationship exists in human tumors.

A model system has recently been described which should expediate this investigation (4). Human malignant cells in tissue culture have been able to produce tumors readily in NIH Swiss mice treated with antithymocyte serum. Epithelial cells derived from human carcinomas produce carcinomas in the mouse, whereas fibroblastic cells from sarcomas yield sarcomas (Paul Arnstein, personal communication). A lung carcinoma and a sarcoma both of which are near diploid have been made available for our initial studies. They produce rapidly growing carcinomas and sarcomas respectively in the mouse (Paul Arnstein, personal communication). We have already used chromosomal changes as criteria for the diagnosis of human malignancy in solid tumors and effusions (5-7). If specific chromosomal changes could be recognized, this would facilitate chromosomal diagnosis of malignancy in humans as well as increase our understanding of etiological factors in human cancer.

Since growing human tumor cells in the mouse has yielded C-type RNA particles in the human cells, whereas growing the cells in newborn hamsters has not thus far (Robert McAllister, personal communication), we shall grow the tumor cells in both of these systems.

B. It is only recently that hamster or rat tracheal tissue has been grown with success in long and short term organ culture without changing morphological and biochemical functions (8,9). It has been shown that H-benzo(a)pyrene binds to DNA more extensively than controls in the presence of F_2O_3 and less in the presence of 7,8-benzoflarone than in controls (8). Also, early metaplastic-like morphological changes have been seen in longer term rat tracheal cultures following exposure to polycyclic hydrocarbons (9). Thus, a similarity to the in vivo situation has been established. We hope to use this similarity to study the relationship of early chromosome changes in the system to the in vivo development of malignancy.

9. Experimental Design:

A. Initially a near diploid long carcinoma cell line and a sarcoma cell line will be studied. Chromosomal analysis will be done before and after producing tumors using the trypsin-Giemsa

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banding technique (10). Human tumors will be established in mice and hamsters by treating the animals with antithymocyte or antilymphocyte serum (4). Pathological diagnosis will be made on the tumors and expression of RNA-virus monitored by the reverse transcriptase assay. Tumors will be placed back in cell culutre. Clones of cells will then be selected which have lower tumor producing potential and these clones will be evaluated for change in chromosome complement from the tumor producing clones . Subsequently all tumor lines isolated at the Childrens Hospital will be evaluated in a similar fashion.

B. Hamster tracheal tissue in organ culture will be exposed to both high dose short term and low dose long term treatment of polycyclic hydrocarbons and ara-C. Simultaneous histological and chromosome studies will be done at various times after exposure, as has been described in part A.

10. Space and Facilities available:

Complete laboratory for karyotyping
Appropriate laboratory equipment
Dark room for developing and printing film.
Adequate animal facilities

11. Additional Facilities Required:

None

12. Biographical sketches of investigator(s) and other professional personnel:

(Appended)

13. Publications:

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First Year Budget:

A. Salaries:

NAME	% TIME	AMOUNT
William F. Benedict, M.D.	20	-0-
Peter Jones, Ph.D.	20	-0-
Hart Isaacs, M.D.	5	-0-
Benjamin Landing, M.D.	5	-0-
Mary Baker	80	10,000
Betty Paul	20	3,000
Corey Mark	20	1,500
	Sub-total for A.	14,500

B. Consumable Supplies:

Antithymocyte and Antilymphocyte serum (3.75/test animal), 2,200
Animals (hamsters, and mice), 1,200
Tissue Culture Dishes, 1,000
Medium and serum, 800
Film and Processing, 400

Sub-total for B. 5,600

C. Other Expenses:

Travel (To Microbiological Associates, Bethesda, Maryland for Principal Investigator).

Sub-total for C. 600

26700.

D. Permanent Equipment:

Laminar Flow Hood, 650
CO_2 Incubator, 1,200

Sub-total for D. 1,850

E. Indirect Costs:

37.62% of salaries and wages per DHEW Agreement dated February 23, 1972. 5,050

3105

TOTAL REQUEST: 27,600

25655.

Estimated Future Requirements:

Year 2	Salaries	Supplies	Other	Equipment	Ind. Cost	Total
	$15,400	$6,000	$600	-0-	$5,353	$27,353

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6. Benedict, W.F., Brown, C.D., Porter, I.H.: Long acrocentric marker chromosomes in malignant effusions and solid tumors. N.Y. State J Med 71:952-955, 1971.

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Other Sources of Financial Support:

CURRENTLY ACTIVE

TITLE OF PROJECT	SOURCE & NUMBER	AMOUNT	INCLUSIVE DATES
Malignant Transformation of DNA Inhibitors	NCI-CA14226-01	$120,700	2-1-73 - 1-31-76
Cytogenetic Diagnosis and Etiology of Human Cancer	NCI-CA15039-01	$75,000	6-30-73 - 8-31-76
Chromosome Changes in Malignancy in the Mouse	Council for Tobacco Research	$10,000	7-1-73 - 6-30-74

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SECTION II – PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
William F. Benedict, M.D.	Assistant Professor of Pediatrics Hematology & Cytogenetics	R
PLACE OF BIRTH (City, State, Country) REDACTED	**PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)**	**SEX** ☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Drew University, Madison, New Jersey	BS	R	Zoology
Albany Medical College, Albany, N.Y.	MD	1968	Medicine
Childrens Hospital of L.A., L.A. Cal.	Intern	1969	Pediatrics
Albany Medical College, Albany, N.Y.	Resident	1970	Pediatrics
NICHD, Bethesda, Maryland	Post. Doc.	1970-72	Genetics & Carcinogenesis

HONORS

Recently named Research Consultant to the Birth Defects Institute of New York State.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Carcinogenesis & Cytogenetics	Principal Investigator

RESEARCH SUPPORT *(See instructions)*

Grant No.	Title of Project
CA-14226	Malignant Transformation of DNA Inhibitors and Cell Cycle Specificity

Amt. for Curr. Yr.	Amt. for Proj. Per.	Time or Effort % Hrs.	Source
$45,900	$120,701	75%	NCI

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)*

For the past two years I have been doing post doctorate training in the Department of Developmental Pharmacology at the National Institute of Child Health and Human Development under the direction of Dr. Daniel Nebert.

My research has included studies in hamster fetal cultures on the relationship of polycyclic hydrocarbon produced cytotoxicity, transformation and chromosomal aberrations (7,8). I have also studied hydrocarbon-produced tumorigenesis in aryl hydroxylase inducible and non-inducible strain of mice and their appropriate hybrids using phorbol ester as a promoting agent (10). Finally I have investigated the genetic expression of aryl hydrocarbon hydroxylase induction in hybrid cells found between inducible and non-inducible parental cells (9,12,13,16).

During my year of pediatric residence at the Albany Medical Center I continued my studies with Dr. Ian Porter, Professor of Pediatrics and Director, Birth Defects Institute of New York State on the cytogenetic diagnosis of malignancy in effusions and solid tumors (1,3,5,6). While a resident I also spent three months at the Children's Hospital of Los Angeles as an Oncology Fellow continuing the work I had begun with Dr. Myron Karon, Head, Division of Hematology, on DNA synthetic inhibitors, cell cycle specificity and chromosomal breakage in hamster cells (2,4,11), while I was a pediatric intern at the Los Angeles Children's Hospital.

All four years as a medical student I spent a significant amount of time with Dr. Porter studying chromosomes in malignant and undiagnosed effusion and solid tumors, showing that cytogenetics could be used as a diagnostic procedure.

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8. Benedict, W.F., Rapid Production of Numerical and Structural Abnormalities in Hamster Cells by Transforming Doses of Polycyclic Hydrocarbons, J. Natl. Cancer Inst., 49, 585-590, (1972).

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11. Karon, M. and Benedict, W.F., Chromatid Breakage: Differential Effect of Inhibitors of DNA Synthesis on G_2 Science, 178, 62, (1972).

12. Benedict, W.F., Paul, B. and Nebert, D.W., Expression of Aryl Hydroxylase in Mouse-Hamster and Mouse-Man Hybrids, Biochem. Biophys Res. Comm., 48, 293-298, (1972).

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LETTERS AND ABSTRACTS

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Benedict, W.F., Gielen, J.E. and Nebert, D.W., Inducible Aryl Hydrocarbon Hydroxylase and 7,12-Dimethylbenz(a)anthracene-produced Skin Tumorigenesis in the Mouse, Amer. Assoc. of Cancer Res., May, 1972 (Abstract #481).

Karon, M. and Benedict, W.F., Comparative Pharmacology of Chromatid Breakage: The Effect of Various DNA Inhibitors, Amer. Assoc. of Cancer Res., May, 1972 (Abstract #202).

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NAME Mary Sherman Baker

ADDRESS

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PHONE

BUSINESS ADDRESS National Institutes of Health
Bethesda, Maryland

BUSINESS PHONE (301) 496-5391

EDUCATIONAL BACKGROUND

High School: Yorktown High School
Arlington, Virginia

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College: William and Mary
Williamsburg, Virginia
1962-1966, B.S. Degree

Graduate School: R R R
Washington, D.C.
1968-1970, M.S. Degree

Chief Undergraduate Subjects:

Biology	33 hours
Chemistry	18 hours
Education	15 hours

Chief Graduate Subjects:

Cell Physiology	23 hours
Biochemistry	10 hours

Master's Thesis: Isolation and Characterization of Nuclear and Cytoplasmic RNA in Cultured and Uncultured Hamster Embryo Cells.

Additional Graduate Courses:
National Institutes of Health Graduate School Program, 15 hours.

HONORS, FELLOWSHIPS

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WORK EXPERIENCE

1. May, 1970 - Present

National Institutes of Health
Bethesda, Maryland

Supervisor: Dr. Michael B. Sporn, Head, Lung Cancer Segment, National Cancer Institute

Salary: REDACTED

Position: Biologist

Description of Work

The Lung Cancer Segment is currently attempting to set up animal systems, both in vivo and in vitro, for studying the biochemical effects of lung carcinogens and anti-carcinogens with the goal of finding a way to both diagnose and treat lung cancer earlier than is currently possible. I have been involved in four separate projects, all of which were centered on this goal.

1.1 Treatment of hamsters with benzopyrene and hematite suspensions intratracheally for the purpose of inducing lung tumors.

My duties and responsibilities were to anaesthisize and administer carcinogen to a large group of hamsters, both controls and test animals. The project lasted for ten weeks after which the hamsters were kept for up to one year before lung tumors developed. I was not involved in the final processing or the tumors.

1.2 Inhibition of HeLa cell RNA exoribonuclease by specific enzyme inhibitors.

The goal of this research project was to find chemicals which would specifically inhibit RNA exoribonuclease activity thus allowing all RNA synthesized in the cell to be used. Presumably, if malignancy were due to a degradation of normally needed RNA, the inhibition of this enzyme would allow the cell to function normally again. I was responsible for growing HeLa cells, isolating nuclei, performing enzyme assays, and tabulating data.

1.3 Establishment of an in vitro method for studying the effects of vitamin A deficiency on RNA synthesis in hamster tracheal epithelium.

This project lasted for approximately one year and was the major effort to which I was devoted during my employment at NIH. The first part of the project involved setting up a means for maintaining viable hamster tracheas in vitro for up to 4 hours for the purpose of incorporation of radioactive precursor at high enough levels to give significant data (this cannot be done in vivo).

The second part of this project involved studying the pattern of RNA metabolism in the tracheas of hamsters which had been made

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deficient in vitamin A. (Vitamin A has been implicated as a possible anti lung carcinogen). I was responsible for incubation and labelling of tracheas, removal of epithelium, extraction of RNA and polyacrylamide gel slab electrophoresis of RNA. A computer program was devised for the analysis of data, and I was responsible for entering data and obtaining results from the computer.

1.4 Isozyme patterns in tracheal epithelium of vitamin A deficient hamsters and hamsters treated with carcinogens.

This is the project which I am currently involved with. Approximately six months have been spent in working out the techniques for separation of the isozymes of lactate dehydrogenase, malate dehydrogenase, aldolase, hexokinase by polyacrylamide gel disc electrophoresis. I have been responsible for obtaining epithelial samples, preparing homogenates, running gels, and staining gels. I have also done some work in attempting to quantitate results. At this point, the techniques for analysis have been worked out and the project will continue to study isozymes in vitamin A deficient tracheas, lung tumors, and carcinogen treated tracheas in hopes of obtaining a biochemical marker for early malignancy and subsequent reversal of malignancy. Tracheal organ cultures will soon be set up to aid in more rapid treatment of tissue, and I will be partially involved in this.

2. February, 1968 - February, 1970

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Washington, D.C.

Advisor: Dr. Lawrence Alfred

Position: Graduate student, NIH Fellow

Description of Work

My graduate work at Catholic University consisted of a full time investigation of RNA metabolism in tissue culture cells. Whole hamster embryos as well as embryo cells in various stages of passage were studied. The work was done at the National Institutes of Health in the laboratory of Dr. C. W. Dingman.

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3. July, 1966 - February, 1968

National Institutes of Health
Bethesda, Maryland

Supervisor: Dr. Joseph DiPaolo, Head, Cytogenetics Section, Biology Branch, National Cancer Institute.

Salary: REDACTED (GS-7)

Position: Biologist

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Description of Work:

The Cytogenetics Section was involved in working out a model for malignant transformation by carcinogen in cultured hamster embryo cells. I worked on a project in which levels of the carcinogen metabolizing enzyme, benzpyrene hydroxylase, were studied over various time periods in synchronized cells treated with benzanthracene. I was responsible for setting up primary cultures and maintaining cell lines, treating cells with chemical carcinogens, measuring radioactive precursor incorporation, and collecting cells for enzyme assays.

TYPE OF POSITION DESIRED

I would like to participate in studies on cellular control mechanisms which would utilize my extensive training and experience in the analysis of macromolecular synthesis and enzyme activity.

Position: Graduate student, Kim Fello

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PUBLICATIONS

The Cytogenetics Section was involved in work on a model [illegible]

1. Protection of normal hamster cells from in vitro initiated cytotoxicity by dimethylbenz (a) anthracene following aryl hydroxylase induction. L. Alfred, P. Donovan, M. Baker, and J. DiPaolo. Cancer Research, 29:1805-09, 1969.

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3. Coordinated biochemical and morphologic examination of hamster tracheal epithelium. D. Kaufman, M. Baker, C. Harris, J. Smith, H. Boren, M. Sporn, and U. Saffiotti. J Natl Cancer Inst 49: 783-792, 1972.

4. RNA metabolism in tracheal epithelium: alteration in hamsters deficient in vitamin A. D. Kaufman, M. Baker, J. Smith, W. Henderson, C. Harris, M. Sporn, and U. Saffiotti. Science, 177: 1105-1108, 1972.

ABSTRACTS

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REFERENCES

1. Mr. Paul Donovan
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Bethesda, Maryland 20014

2. Mr. Milford Greene
Wesleyan University
Department of Biology
Middletown, Connecticut

3. Dr. Richard DeCicco
Catholic University
Department of Biology
Washington, D.C.

4. Dr. Lawrence Alfred
Federal City College
Department of Biology
Washington, D.C.

5. Dr. C. Wesley Dingman
National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20014

6. Dr. David Kaufman
National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20014

7. Dr. Michael Sporn
National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20014

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CURRICULUM VITAE

REFERENCES

BENJAMIN H. LANDING, M.D.

Business Address	4650 Sunset Blvd., Los Angeles, Calif. 90027
Business Telephone	(213) 663-3341 Ext. 426
Home Address	REDACTED
Home Telephone	
Birth Date	
Birth Place	
Citizenship	
Marital Status	

EDUCATION:

College	Harvard College, A.B., 1942
Medical School	Harvard University Medical School, M.D., 1949
Internship	Pathology, Childrens Hospital, Boston, 1945-1946
Residencies	Assitant Resident, Pathology, Childrens Hospital, Boston, 1946, 1948 Resident, Pathology, Childrens Medical Center, Boston, 1948-1949 Resident, Pathology, Free Hospital for Women and Boston Lying-in Hospital, 1949
Licensure	Massachusetts, 1950 Ohio, 1954 California 1962

Professional Background:

Academic Appointments	Assistant Instructor and Associate in Pathology, Harvard University Medical School, 1945-1953
	Lecturer in Embryology, Boston University Postgraduate School of Nursing, 1949-1952
	Assistant Professor, Pathology and Pediatrics, University of Cincinnati College of Medicine, 1953-1958
	Associate Professor, Pathology and Pediatrics, University of Cincinnati College of Medicine, 1958-1961

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BENJAMIN H. LANDING, M.D. CON:

PROFESSIONAL BACKGROUND CON:

Academic Appointments	Professor of Pathology and Pediatrics, University of Southern California School of Medicine, 1961 - present
Hospital Affiliations	Assistant Pathologist, Childrens Medical Center, Boston, 1950-1953 Histochemist, Childrens' Cancer Research Foundation, Boston, 1950-1953 Directing Pathologist, Children's Hospital and Children's Hospital Research Foundation Cincinnati, 1953-1961 Pathologist-in-Chief and Director of Laboratories, Childrens Hospital of Los Angeles, 1961-present
MILITARY SERVICE:	First Lieutenant and Captain, M.C., AUS Medical Research Labs, Army Chemical Center, Maryland, 1946-1958

SOCIETY MEMBERSHIPS:

Local

National

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RESEARCH ACTIVITIES AND INTERESTS (in part):

Histochemistry of endocrine metabolic and genetic diseases.
Nosology of genetic diseases, especially of kidneys, nervous system, immune system, skeleton.

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Benjamin H. Landing, M.D.

PROFESSIONAL BACKGROUND

Landing, B. H.: Parasitic carcinogenesis. Boylston Soc. Reports, 1944-45

Krop, S., Wescoe, W. C., Goldin , A., Landing, B.: Central nervous system injury in experimental animals by betacholroethyl morpholine. Fed. Prpc., Vol 6, No. 1, March, 1947. (ABSTRACT)

Karel, L., Landing, B. H., and Harvey, T. S.: The intraperitoneal toxicity of some glycol ethers, glycol esters, and phthalates in mice. J. Pharm. Exp. Ther., Vol. 6, No. 1, March, 1947.

Goodale, W. T., Lubin, M., Eckenhoff, J. E., Hafkenschiel, J. F., Durlacher, S.H., Landing, B. H., and Banfield, W. G.: Coronary sinus catheterization: Technique for studying coronary blood flow and myocardial metabolism in vivo. Proc. Soc. Exp. Biol. Med., 66:571-573, 1947.

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Landing, B. H., Seed, J. C., and Danfield, W. G.: The effects of nitrogen mustard (Tris-2-Chlorethyl amine) on regenerating rat liver. Cancer, 2:1067-1074, Nov., 1949.

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Blysted, W., Landing, B. H., and Smith, C. A.: Pulmonary hyaline membranes in newborn infants. Statistical, morphologic and experimental study of their nature, occurrence and significance. Pediatrics, 8:21, 1951.

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Nadas, A., Cogan, G., Landing, B. H., and Shwachman, H.: Studies in pancreatic fibrosis. Cor pulmonale: clinical and pathologic observations. Pediatrics, Vol. 10, No. 3, Sept., 1952.

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Landing, B. H.: Pathology of fibrocystic disease of the pancreas - in Grulee and Eley, The Child in Health and Disease, 2nd edition, pp. 736-738, 1952.

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F. Research Activities

1. Bibliography and reprints attached
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J. of Urology, Vol. 104, Oct., 1970

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J. Pediat. Surg., Vol. 7, p. 517 (1972)

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Arch. Surg., Vol. 106, p. 507, (1973)

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#935 - FISHMAN

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

August 6, 1973

Grant Application No. 935
CANCER

To: The committee comprising Drs. Andervont, Huebner, and Jacobson

Subject: William H. Fishman, Ph.D., Tufts Cancer Research Center
New application No. 935
"Embryonic Gene Activation in Bronchogenic Cancer"

History

An initial informal inquiry from Dr. Fishman was in terms of possible broad institutional support for his group. Dr. Lisanti was asked to visit, to discuss a specific proposal. He saw Dr. Fishman several times and the enclosed application resulted.

Application No. 935 requests $62,853 plus two additional years.

Documents Submitted (attached)

1. Letter to Dr. Gardner dated July 30, 1973.
2. "SUMMARY STATEMENT".
3. Application dated 7/30/73.
4. "APPENDIX"
5. C.V.'s of Drs. Fishman, Chang, Doellgast, Gandbhir, Miyayama, Raam, and Stolbach.
6. Lists of recent publications, Drs. Fishman, Stolbach, and Chang.
7. Smoking Questionnaire.
8. Drinking Questionnaire.

Comment

Reprints of pertinent publications were provided, and will be forwarded if you request.

F.W.N.

FWN:wg
Encls.

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TUFTS CANCER RESEARCH CENTER
OFFICE OF THE DIRECTOR

TUFTS UNIVERSITY
SCHOOL OF MEDICINE

136 HARRISON AVENUE
BOSTON, MASSACHUSETTS 02111

July 30, 1973

Dr. William U. Gardner, Chairman
Scientific Advisory Board
The Council for Tobacco Research
110 East 59th Street
New York, New York 10022

Dear Dr. Gardner:

I am pleased to submit with this letter a grant application entitled "Embryonic Gene Activation in Bronchogenic Cancer".

From the general point of view, it represents an experimental approach to cancer which is based on rather new information which began to appear in the literature since 1965. At first, it was α-fetoprotein which is a fetal gene product recognized in human hepatoma. Then, in 1967, a protein of the fetal gastrointestinal tract was discovered in human gastrointestinal cancers (CEA). Shortly afterward, the Tufts group encountered in lung cancer the Regan isoenzyme which is a placental alkaline phosphatase. Most recently, human chorionic gonadotrophin, another placental protein, has been identified in a variety of non-trophoblastic tumors. The literature now reflects a broadening interest and enthusiasm for the study of embryonic gene products in human cancer and their significance.

Our own experiences with the Regan isoenzyme have prepared us for systematic studies of a number of these carcinoembryonic proteins. These experiences have included the preparation of pure placental alkaline phosphatase, the study of its phenotypic forms, the discovery and application of specific amino acid inhibitors to its quantitation and histochemical demonstration, the development of ultrasensitive immunoenzyme techniques for its detection and, finally, the completion of extensive clinical studies designed to evaluate its usefulness in prognosis and differential diagnosis. These have all been accomplished in four years after the Regan isoenzyme was discovered.

Specifically, with regard to cancer of the lung, we are challenged to find out what results could be harvested from the application of knowledge in the area of embryonic gene activation. We think that the opportunity to examine biochemically, cytologically, and histochemically the bronchial epithelium geographically in relation to a metaplastic, cancer in situ or a frankly neoplastic area is unique and could elicit the type of information which is truly meaningful. We are hopeful that once we have a basic under-

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standing of the role of embryonic gene activation, a logical approach to certain fundamental questions relative to lung cancer can be fashioned.

Should your committee look with favor to supporting our plan of research, the study of cancer of the lung would become a major commitment of all the professional, technical and physical resources of the Tufts Cancer Research Center. It would provide a sharply focused goal towards which the cancer cell phenotype group would orient itself. This, in turn, will be bound to stimulate a lot of research interest in specific problems which would be defined as the project moves ahead.

May I point out that the Faculty identified with the Center is competent in the areas of molecular biology, immunology, isoenzymology, somatic cell genetics, immunoviral oncology and chemotherapy. Accordingly, we are confident of the potential of this group to tackle almost any aspect of the contemporary oncology scene if the interest generated from the proposed research warrants it.

In closing, I wish to express the hope that you may find the application of substantial interest to you and your colleagues and also that you may find yourselves able to provide us the financial support we need to do the job.

I send my best personal regards.

Yours sincerely,

Bill Fishman

William H. Fishman, Ph.D.
Director, Cancer Research Center

WHF:sn
Encl.

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SUMMARY STATEMENT

RESPONSIBLE INVESTIGATOR - William H. Fishman, Ph.D.

INSTITUTION - Tufts Cancer Research Center, Tufts University School of Medicine, Boston, Massachusetts

TITLE OF PROJECT - Embryonic Gene Activation in Bronchogenic Cancer

The basic premise of this multidisciplinary three year program is that a fundamental manifestation of neoplasia is the activation of embryonic, placental and fetal genes. The questions which arise from this premise are (1) to what extent is bronchogenic carcinoma associated with the activation of embryonic genes with respect to four typical carcinoembryonic proteins? (2) Does the individual histologic type of bronchogenic cancer [squamous cell carcinoma, small cell carcinoma, adenocarcinoma, large cell anaplastic carcinoma, mixed tumors] and its degree of differentiation correlate with one or a combination of carcinoembryonic proteins - in the primary lesion? in the metastasis? (3) Do carcinoembryonic proteins appear in the bronchial epithelium of individuals destined to develop bronchogenic carcinoma as studied in metaplastic and in situ carcinoma tissue? (4) Is there a correlation between the smoking and drinking histories of patients and the activation of embryonic genes?

We are in a position to approach these questions experimentally because we have had extensive experience with the Regan isoenzyme [a placental alkaline phosphatase first found in a patient (Peter Regan) with metastatic cancer of the lung] and we have been developing new knowledge through the study of cancer cell phenotypes produced in ovarian cancer, which is the first type of cancer we have been studying.

Cancer of the lung would become the second type of cancer to which the Tufts Cancer Research Center with its basic scientists and clinicians is prepared to commit itself. The funds requested will be the sole initial support of this effort and will make possible not only the radioimmunoassay capability for carcinoembryonic proteins and the participation of the Pondville investigators and their resources but will also provide a forward impetus to the entire research program of the Center.

Attention will be focused on the epithelial elements of the human lung, the recognition and evaluation in the epithelium of embryonic gene expression, the possibility of finding correlations of such findings with either susceptibility or potential for neoplastic progression.

The embryonic proteins include Regan isoenzyme, human chorionic gonadotrophin, CEA and α-fetoprotein which can be determined quantitatively by radioimmunoassay. In addition, we may soon be in a position to demonstrate these proteins cytologically at the level of both the light and electron microscopes. This would enable us to make more precise interpretations of the quantitative values.

With the professional and patient resources at the Pondville Hospital together with the expertise of the scientists in the laboratories of the Tufts Cancer Research Center, the commitment is to research of high objective quality in a multidisciplinary effort. A balance will be maintained between qualitative studies of biologic phenomena and subsequent quantitative evaluations.

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Comm.
Dr. Andervont
Dr. Huebner
Dr. Jacobson

ack 4/23
#935

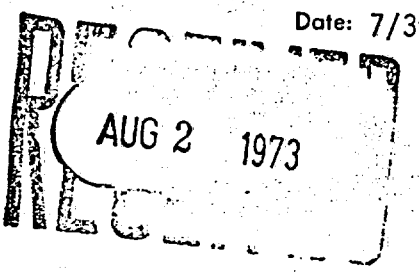
CANCER

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 7/30/73



1. Principal Investigator (give title and degrees):

William H. Fishman, Ph.D.
Director, Tufts Cancer Research Center
Professor of Pathology
Tufts School of Medicine

2. Institution & address:

Tufts Cancer Research Center
Tufts University School of Medicine
136 Harrison Avenue
Boston, Massachusetts 02111

3. Department(s) where research will be done or collaboration provided:

Department of Pathology
Department of Medicine

4. Short title of study:

Embryonic gene activation in bronchogenic cancer

5. Proposed starting date: October 15, 1973

6. Estimated time to complete: three years

7. Brief description of specific research aims:

The specific research aims are directed toward obtaining at least partial answers to the following questions:

a. To what extent is bronchogenic carcinoma associated with the activation of embryonic genes with respect to four typical carcinoembryonic proteins?

b. Does the individual histologic type of bronchogenic cancer correlate with one or a combination of carcinoembryonic proteins - in the primary lesion? in the metastases?

c. Do carcinoembryonic proteins appear in the bronchial epithelium of individuals destined to develop bronchogenic carcinoma as studied in metaplastic and in situ carcinoma tissue?

d. Is there a correlation between the smoking and drinking histories of patients and the activation of embryonic genes?

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8. Brief statement of working hypothesis:

We believe that a fundamental manifestation of neoplasia is the activation of embryonic, placental and fetal genes while the genes characteristic of adult tissue may continue to be expressed or to a certain extent, suppressed. It is conceivable that the carcinoembryonic genes may, in certain combinations, be responsible for the cell acquiring its autonomous character. For example, placental trophic hormones such as human chorionic gonadotopins, placental lactogen, and thyrotropic hormones, are products of and may have stimulatory effects in a variety of cancers. Conceivably, therefore, although some of the gene products cannot be seen to have any particular relevance to the autonomy of the cancer cell, others do. It is imagined that amongst the first events in a cell undergoing neoplastic change should be the activation of several embryonic genes. On this basis, it may be possible to identify individuals whose ability to undergo activation of embryonic genes in their own cells is greater than it is in other individuals. This would give us some means of evaluating susceptibility or resistance to neoplasia. In the case of cancer of the lung, the present circumstances favor the study of the appearance of embryonic genes in bronchial epithelium of individuals with and without bronchogenic cancer. It is also possible that a knowledge

(contd. on separate sheet)

9. Details of experimental design and procedures (append extra pages as necessary)

Details of experimental design and procedures

The project can be visualized as developing in two stages; first, the "phasing-in" process and second, the initiation and operation of the fully organized study.

"Phasing-in"

We would plan to test the adequacy of our tissue and cell sampling techniques, the completeness of extraction of the various carcinoembryonic proteins and the reproducibility of the radioimmunoassay procedures on specimens of bronchogenic cancer, bronchial epithelium, etc., which we have available in cold storage or which present themselves at surgery.

Similarly, the pathologist would develop and standardize the practices which will be the most optimal in order to ensure selection of representative specimens, the labeling techniques which would be the most foolproof and the establishment of a routine from beginning to end with a concern for objectivity in reporting the histopathologic findings.

Obviously, the scope of the study bears some relation to the autopsies and surgicals to be performed. A measure of this facet is provided by the following table.

PATHOLOGICAL MATERIAL - PONDVILLE HOSPITAL

Site of Cancer	New Patients	1972 Autopsies	1973 Autopsies	1974 Autopsies
Lung	65	26	PROJECTED	PROJECTED
Breast	117	19		
Colon	43	5		
Prostate	22	3		
Bladder	12	3		
Stomach	7	0		
Other	428	43		
	694	99	109	120

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8. Brief statement of working hypothesis (contd.)

of the nature and extent of embryonic gene expression in different types of bronchogenic carcinoma could be valuable in estimating the prognosis in individual patients. Since these embryonic gene products are normally foreign to the host and since they are able to get into the bloodstream, the potential for serodiagnosis by radioimmunologic measurements exists. The resulting information could give the physicians an indication more broadly based than on the H and E section alone as to the "activity" of the bronchogenic cancer and could provide an objective means of monitoring the disease.
We have preliminary evidence that in a population of alcoholics the Regan isoenzyme is expressed in a high percentage of cases. [In this connection, we have been wondering about the significance of an alcoholic patient whose serum was Regan-positive and two years later developed an undifferentiated cancer of the lung.] If individuals who consume alcohol have a predilection to activate embryonic genes, then these individuals may become high risks for bronchogenic, and head and neck cancers if atmospheric irritants represent an additional contributing factor for activating the embryonic genes further.

With the availability of immunohistochemical techniques, it should be possible to identify the individual cells in the normal and malignant bronchial epithelium which are expressing the various embryonic gene products. These would include Regan isoenzyme, CEA, HCG, and α-fetoprotein.

This working hypothesis has developed from our own studies on the Regan isoenzyme (placental-type alkaline phosphatase) in cancer and non-cancer patients (see reprints) and from the experiences of others with CEA and α-fetoprotein. We have found that abnormal amounts of Regan isoenzyme were present in the serum of one out of seven patients and in a number of conditions predisposing to or associated with pre-neoplastic situations (Nathanson, L., and Fishman, W.H., Cancer, 27: 6, 1388-1397, 1971; Stolbach, L.L., Krant, M.J., and Fishman, W.H., N.E.J. Med., 281: 757-762, 1969). With the development of ultrasensitive techniques, we are now finding a greater percentage of cancer patients whose serum is Regan-positive along with an appreciable incidence of trace amounts in individuals who are presumably healthy. This experience fits the results others are reporting for CEA and α-fetoprotein and suggests that embryonic gene activation is taking place in cells of normal individuals at a low rate and that the appearance of higher titers in malignancy represents a quantitative rather than a qualitative manifestation.

The choice of four carcinoembryonic proteins (Regan isoenzyme, HCG, CEA, α-fetoprotein) is not meant to exclude other proteins which are not proven carcinoembryonic as yet but which are now beginning to attract attention. For example, Gewirtz and Yallow at the 55th Annual Meeting of the Endocrine Society reported that big and little forms of ectopic ACTH can be distinguished and that it is the former which is immunoreactive but not biologically active. Amongst other finding, they state that five of five bronchogenic carcinomas and one bronchial adenoma obtained at surgery contained ACTH, while none was detectable in two surgical specimens of normal human lung. Also, in our laboratory, we are examining a varity of placental enzymes and proteins with a view to identifying additional carcinoplacental proteins. As soon as this is successful, we would plan to include measurements of these additional proteins in the lung cancer study.

The relationship of the activation of embryonic genes as compared to activation of oncogenes still remains to be established. It is our hope that success in the work projected will be helpful in the examination of the oncogene hypothesis which is the challenging theory of neoplasia today.

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9. Details of experimental design and procedures (contd.)

In our opinion, there is more than an adequate supply of suitable cases (60% autopsy rate of patients dying at Pondville Hospital) and our habit is to carry out as thorough and complete studies as possible. Also, since Pondville Hospital accommodates cancer patients only, the comparisons initially will be the bronchial epithelium and tumors of lung cancer patients versus the bronchial epithelium of patients with cancer at other sites.

Tumor tissue will be processed for phenotypes in the case of the non-lung cancer patients whose bronchial epithelium is included iin the study.

HISTOLOGIC CLASSIFICATION OF LUNG CANCER

1. Squamous cell carcinoma, well, moderately or poorly differantiated

2. Small cell carcinoma (including oat cell)

3. Adenocarcinoma (including bronchiolar), well, moderately, poorly differentiated

4. Large cell anaplastic carcinoma (undifferentiated carcinoma)

5. Mixed tumors will be classified according to the predominant cell element and other types will be stated

Pathologic Study

It will be necessary to establish the number of samples to be taken from a given area of bronchial epithelium to give reproducible results. In the event abnormal epithelium is visible, cell suspensions of abnormal epithelium will be made separately from those of normal-appearing epithelium. In addition, some blocks of the trachea will be submitted to routine hematoxylin and eosin staining for a point of reference.

At autopsy we will obtain the distal 3 cms of trachea, right and left main stem bronchi to the point of bifurcation. Tissue will be taken for histology from (a) the proximal end of trachea, (b) the carina, (c) at the bifurcation of main stem bronchi.

This will be standard procedure in control cases as well as carcinoma of lung, presuming the pathologic process has not infiltrated bronchi.
If there are pathologic lesions involving the above areas, we would get as much normal mucosa as possible, 2-3 cms from the lesions from either bronchi or trachea.
In cases of carcinoma of lung, we will collect tissue where it appears most viable, preferably in the following order:

a. primary site
b. regional lymph nodes
c. metastases to distant organs

A minimum of two sections per block will be processed histologically for this particular project with blocks and slides, which will be filed separately.

Collection of bronchial epithelial cells

Several alternatives are under consideration.

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9. Details of experimental design and procedures (contd.)

One possibility is to dissect out the mucosal epithelium and to check the completeness of its removal by histologic examination of the remaining tissue. Another approach is to use the technique of Dr. B. Spencer, published in the Journal of Histochemistry and Cytochemistry, 6: 105, 1958. In this technique, autopsied specimens of bronchus and trachea are trimmed of lymphatic and fatty tissue and cut into pieces about 2 cm square. Superficial mucous is removed by lightly brushing the surface of the epithelium with a No. 5 camel hair brush in the presence of the liquid medium at 0°. A portion of the epithelium is then isolated by pressing onto it a lucite tube of 1 sq. cm cross sectional lumen area so as to form a water-tight seal. Liquid medium (0.2 ml) is added into the tube and the epithelium brought into suspension using a No. 2 lettering brush with the bristles trimmed to a length of 4 mm. The most satisfactory results are obtained by twirling the brush between the thumb and forefinger and maintaining a very light downward pressure. The suspension is removed with a transfer pipette and the process repeated with two further 0.2 ml portions of the liquid medium and the suspensions combined. The epithelial suspension so prepared consists of clumps of cells which are broken up by repeated passage through a pipette with an orifice of 0.1 - 0.2 mm. A portion of the suspension is retained for cell counting and the remainder distributed among a number of small tubes which are capped with parafilm and stored at -20° until required for biochemical assay. With this technique, normal epithelia of both bronchus and trachea possess between 2 and 3 million cells per sq. cm and a ratio, intermediate:goblet:ciliated:basal cells appears to be of te order of 0.2:0.4:1.0:1.6 and the ratio, columnar:basal cells is approximately 1:1. The chief value of this technique is that it provides a means of relating the chemical properties of epithelium to the cytological composition of th tissue since assays for carcinoembryonic proteins and differential and total cell counts are made on the same suspension.

Determination of carcinoembryonic proteins

Once it has been established how the tissue specimens and cell suspensions can be aliquoted and best preserved for the subsequent assays and best processed to release all embryonic protein into solution, the following techniques will be employed.

(a) the proximal end of trachea, (b) the carina, (c) at the bifurcation of main

Regan Isoenzyme. This alkaline phosphatase will be extracted and assayed according to the procedures used originally to accomplish this purpose (Fishman, W.H., Inglis, N.R., Stolbach, L.L. and Krant, M.J., Cancer Res., 28: 150-154, 1968). The confirmation of Regan isoenzyme as a placental alkaline phosphatase will be made by Ouchterlony double-diffusion techniques (Fishman, W.H., Inglis, N.R. and Green, S., Cancer Res., 31: 1054-1057, 1971) and by microzone membrane electrophoretic technique (Inglis, N.R., Guzek, D.T., Kirley, S., Green, S. and Fishman, W.H., Clin Chim Acta, 33: 287-292, 1971). The detection of the Nagao type of isoenzyme will be made on the basis of L-leucine inhibition (Inglis, N.R., Kirley, S., Stolbach, L.L. and Fishman, W.H., Cancer Res., 33: 1657, 1973). The results will be expressed as indicated in Placental isoenzyme units using an appropriate term such as, per gram of tissue or per million cells or per mg DNA.

HCG - Human Chorionic Gonafotropin. We intend to use the radioimmunoassay which specifically measures human chorionic gonadotropin in the presence of human luteinizing hormone (Vaitukaitis, J.L., Braunstein, G.D. and Ross, G.T., Am. J. Obstet. Gynecol., 113: 751-758, 1972). In this assay, tracer quantities of the beta subunit of human chorionic gonadotropin (HCG-β) is iodinated with ^{125}I by the chloramine-T method to a specific activity of 50 to 100μCi/ug. The reference preparation is a highly purified HCG preparation. Two hundred microliters of serum or plasma from patients is assayed and the same amount of outdated male

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9. Details of experimental design and procedures (contd.)

blood bank plasma is added to all background and standard tubes to obviate a non-specific plasma protein effect. The tubes are incubated for 2 hours at 37°C and then for an additional 15 to 17 hours at 4°C. Then the sheep anti-rabbit serum is added and the incubation is continued for an additional 6 to 12 hours at 4°C. Separation of bound and free hormones by centrifugation is followed by measurement of the bound radioactive trace in a gamma counter. The within assay coefficient of variation is 15% and the between assay coefficient of variation is 27% for four replicate samples that range from 7.3 to 19.7 mg/ml.

CEA - Carcinoembryonic Antigen of Gold. The original radioimmunoaasay method of Thomson, D.M.P., Krupey, J., Freedman, S.O. and Gold, P., Proc. Natl. Acad. Sci., 64: 161-167, 1969, requires 5 ml of serum which is extracted with perchloric acid. Following centrifugation, dialysis, lyophilization, resuspension in antibody-containing solution, the mixture is incubated with labeled antigen and the bound labeled antigen separated by centrifugation of its ammonium sulfate solution. We are attracted to the recently published radioimmunoelectrophoretic binding assay of Coller, J.A., Crichlow, R.W., and Yin, Lo Ke, Cancer Res., 33: 1684-1688, 1973, which involves simultaneous electrophoretic binding and separation with the use of 10µl of whole sera and requires only two hours.

α-Fetoprotein. Radial immunodiffusion techniques such as those of Abelev (Cancer Res., 28: 1344-1350, 1968) and of Alpert, M.E., Uriel, J. and DeNechaud, B. (New Eng. J. Med, 278: 984-986, 1968) are standard methods for measuring α-fetoprotein in serum.

More sensitive and rapid techniques are now available. The one which appeals to us in the electroimmunodiffusion method of Sizaret, P.P., McIntire, K.R., and Prinder, G.L. (Cancer Res., 31: 1899-1902, 1971) in which a linear relationship is established between antigen concentration and length of precipitin (Ag-Ab) peak. Especially when the antiserum is radioiodine-labeled, the sensitivity of the test is greatly increased.

Clinical Study

Each patient will receive a physical examination on admission and a blood specimen would be collected for the assay of carcinoembryonic proteins. Questionnaires (examples attached) will be filled out by the patient regarding the history of smoking and of drinking. Abstract of pre-admission medical history and a synopsis of the patient's history while in the hospital will be prepared separately for each patient. Since one cannot know in advance which patients will come to autopsy, it will be necessary to evaluate every admission.

Information from smoking and drinking histories plus the histologic diagnosis of the tumor plus the laboratory data on carcinoembryonic proteins should provide sufficient data on which to base a decision whether or not to expand the study to other populations of patients. These non-cancer subjects are readily available at the Shattuck and New England Medical Center Hospitals, institutions which are an integral part of the operation of Tufts Cancer Research Center.

Finally, the team would meet at regular intervals to evaluate their individual roles, to work out problems in coordination of effort and to decide on the best way to collect and analyze the information from both qualitative and statistical points of view.

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9. Details of experimental design and procedures (cont.):

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Our facilities at Tufts University School of Medicine occupy the entire 4th floor of the South Cove building and include a research area of over 4,000 square feet. Tufts University provided the financial resources for the complete renovation and construction of the Cancer Research laboratory. In addition, the Massachusetts Division of the American Cancer Society made a grant to equip the laboratory with several of the most modern pieces of essential equipment. A brief description of the new facilities and equipment follows.

We have a modern cold room laboratory equipped with air, vacuum, electricity and water. In addition, there is sufficient space to house two large fraction collectors equipped with two UV monitors with two recorders. A separate service room is available which houses a Heinicke dishwasher completely overhauled and updated, well insulated steam drying ovens, a small autoclave as well as a large sink for manual glassware washing. There is also sufficient shelf space to store all essential items needed for the routine housekeeping of the laboratory. A solvent storage closet has also been provided. One major facility is the electron microscopy room separate from the EM power supply room, and an adjacent dark room equipped with a 7' photography sink and a Simmons Omega enlarger capable of producing 8" by 10" enlargements. The EM room houses a new JEOLCO 100B transmission electron microscope. The entire suite is isolated from the main laboratory and is designed to maintain proper constant temperature and humidity at all times. In addition, there is an electron microscopy preparation room with an LKB ultramicrotome. Our facilities also include a tissue culture room complete with an Edgeguard laminar flow hood and a large National tissue culture incubator. This room has constant temperature humidity, absolute air filtration and a germicidal light. The instru-

11. Additional facilities required:

None.

the test is greatly increased

each patient will receive a physical examination on admission and a blood specimen would be collected for the assay of carcinoembryonic protein. Questionnaires

information from smoking and drinking histories plus the histo

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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10. Space and facilities available (contd.)

ment room also maintains constant humidity and houses the Beckman DKI, DU, the Coleman 44, Mettler balances, Cahn micro electric balance, plus other items. This instrument room houses previously purchased equipment at the present time. The special photography room is equipped with a Polaroid MP-3 camera plus an air incubator and a Chromatovue Fluorescent viewing cabinet, all of which are employed to develop, observe and photograph the different alkaline phosphatase isoenzymes either by employing cellulose acetate membrane electrophoresis with their detection subsequently with a fluorogenic substrate or by using starch gel electrophoresis with a chromogenic-dye coupler substrate.

The main research laboratory area occupies approximately 3,000 square feet and it is fully air conditioned. The laboratory is sub-divided into areas specifically designed to perform automated enzymology and other related methods, manual enzymology, all types of electrophoresis, protein purification, immunology, histochemistry and light microscopy and radioisotope studies. In addition, we have the use of the Medical School's animal farm which is easily accessible to us for housing our experimental animals. The general laboratory has a 7' radioisotope hood and a 4' fume hood. All laboratory benches have small undercounter refrigerators and freezers for short term use. We have also acquired a Spinco L-2 65B ultracentrifuge, two Sorvall RC-2 centrifuges and a Sorvall GLC-1 centrifuge. These items, in addition to the Nuclear Mark I isotope counter and other centrifuges, are housed in the general laboratory. A separate open storage area distinct from the general laboratory houses the new Kelvinator -76° freezer used for antigen and antiserum storage, the Scotsman ice machine, the Virtis lyophilizer and shell freezer, in addition to several other large freezers and refrigerators.

Finally, communication between our staff is maintained by means of a conference room equipped for oral, written and slide presentations. Adjacent to the conference room is the Director's office. A secretarial office and an office for Sidney Green, Senior Chemist and laboratory manager, completes the administrative suite.

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14. First year budget:

A. Salaries (give names or state "to be recruited")		% time	Amount
Professional (give % time of investigator(s) even if no salary requested)			
W. H. Fishman, Ph.D.	Responsible Investigator	25	*
L. L. Stolbach, M.D.	Co-Investigator	10	3,500
L. Gandbhir, M.D.	Co-Investigator	15	5,000
C. H. Chang, Ph.D.	Co-Investigator	100	15,000
S. Raam, Ph.D.	Research Fellow	30	3,000
H. Miyayama, M.D.	Research Fellow	25	*
G. Doellgast, Ph.D.	Research Fellow	25	*
Technical			
M. L. Orcutt, M.S.	TCRC Lab Res. Asst.	100	9,000
to be recruited	Pondville Res. Asst.	100	8,000
Fringe benefits on all salaries			5,655

*Institutional Funds

Sub-Total for A: 49,155

B. Consumable supplies (by major categories)

Plastic ware, reagents for radioimmunoassay, reagents for tissue purification, glassware, histochemical and histological supplies

okay gk

Sub-Total for B: 5,000

C. Other expenses (itemize)

Travel between Pondville and the Cancer Research Center in connection with pickup of biological materials

Sub-Total for C: 500

Running Total of A + B + C: 54,655

D. Permanent equipment (itemize)

None.

Sub-Total for D: 0

E. Indirect costs (15% of A+B+C): 8,198

Total request: 62,853

15. Estimated future requirements: Res. Asst. at Pondville starting 02 year at 9,040; 7% annual salary increases

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	61,636	6,000	600	0	10,235	78,471
Year 3	65,951	6,000	600	0	10,883	83,434

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Career Award	National Cancer Institute 4K06CA18453-11	29,540	7/1/73 - 6/30/74
2. Fundamental Enzymologic Studies Applied to Oncology	NCI - 7R01CA13332-02	84,020	1/1/73 - 12/31/73
3. Tufts Cancer Research Center	NCI - CA 12924-02	8,727	12/1/72 - 11/30/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Fundamental Enzymologic Studies Applied to Oncology	NCI - 7R01CA13332-03	79,506	1/1/74 - 12/31/74
2. Renewals of other above grants are planned			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name William H. Fishman, Ph.D.

Signature William H. Fishman Date 7/30/73

Telephone 617 (Area Code) 423-4600 (Number) 523 (Extension)

Checks payable to

Tufts University School of Medicine

Mailing address for checks

136 Harrison Avenue

Boston, Massachusetts 02111

Responsible officer of institution

Typed Name Joseph J. Byrne, Ph.D.

Title Research Coordinator

Signature Joseph J. Byrne Date 7/30/73

Telephone 617 (Area Code) 423-4600 (Number) 428 (Extension)

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

W. H. Fishman, Ph.D.
Tufts Cancer Research

APPENDIX

Question

1. Background information
   a. Personal
   b. Tufts Cancer Research Center
   c. Cancer Cell Phenotype Program
   d. Scientific Staff (new)
2. Institutional Commitment
   a. Physical Plant
   b. Faculty Tenure
   c. Operating Expense
3. Departmental Resources
   a. Pathology
   b. Medicine
   c. Pondville Hospital
4. Relationship to Direction of Total Research Effort of TCRC

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Source: https://www.industrydocuments.ucsf.edu/docs/

1. Principal Investigator - Background

a. Personal

The principal investigator has completed 25 years as a Professor of Tufts University School of Medicine, during which time he has been identified with cancer research and cancer teaching. In 1960, his professional competence was recognized amongst other awards by a lifetime NCI Career Professorship Award. He has maintained a balance between fundamental research and clinical application and has been successful in developing and holding together a team of Research Assistants and Faculty collaborators. It was this Cancer Research Laboratory which discovered the Regan isoenzyme (a placental alkaline phosphatase) in a patient with cancer of the lung and had earlier devised techniques for fractionating serum alkaline and acid phosphatases according to their principal organ source. A continuing interest in acid hydrolases (β-glucuronidase, and acid phosphatase) in human tumors and in hormone induction of enzyme activity is evident. As editor, he has just published through Academic Press, Volume 3 of "Metabolic Conjugation and Metabolic Hydrolysis."

With regard to his major research commitment, he has been invited to help organize the "International Research Group for Carcinoembryonic Proteins" at a Symposium to be held in Sapporo, Japan, October 22-25, 1973.

b. Tufts Cancer Research Center - TCRC

This was established January 1, 1972 by virtue of an award by the National Cancer Institute to establish a multidisciplinary Cancer Research Center at Tufts University School of Medicine. The scientific mission of this Center is to expand knowledge in the area of Cancer Cell Phenotypes, and to identify and utilize those of real clinical utility. This mission is what makes the TCRC unique.

As a new entity at Tufts University School of Medicine, the Cancer Research Center does have a position of leadership within the Tufts - New England Medical Center Complex because of its entirely interdepartmental program. It has the support of the departmental Chairmen who have helped to integrate it into the organization of the Medical School, the New England Medical Center Hospital and the other Tufts hospitals. Especially relevant is the wide participation of the Faculty in cancer teaching. Next, leadership within the Cancer Research Center is centered on the Director, who is given the responsibility for the management of the Center and the authority necessary for the effective performance of his duties. He, in turn, operates as a member of the Executive Committee which establishes the overall institutional policies of the Center.

In the area of clinical research and cancer training, three Tufts hospitals are already working closely together as members of the Eastern Cooperative Oncology Group. They are the New England Medical Center Hospital, Pondville Hospital and Lemuel Shattuck Hospital.

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Source: https://www.industrydocuments.ucsf.edu/docs/nfcm0000

1. b. (contd.)

In addition, a CRC bed unit at the New England Medical Center Hospital is to be the focal point for the application of research findings in cancer biology to clinical cancer therapy.

Aside from the role of the Center at Tufts itself, its scope extends to many parts of Boston and to three New England states. Thus, the Boston area is covered by NEMCH and six other Tufts hospitals; Massachusetts, New Hampshire and Rhode Island are included in the tri-state Regional Medical Program, with which Tufts is associated.

For further details, please consult reprint entitled "Tufts Cancer Research Center Opened and Dedicated May 24, 1972".

c. Cancer Cell Phenotype Program

By cancer cell phenotypes, we refer particularly to those which are of greatest interest and relevance to cancer as will be described in the following.

Clinical symptoms or laboratory findings in a cancer patient can often be explained as resulting from the expression of traits or phenotypes of the cancer cell. These phenotypes can be classified into two groups: those which are the gene product similar to that of cells from which the tumor originated (expected) and those which are the gene product different from that of cells from which the tumor originated (unexpected or "ectopic").

Examples of the "expected" category are the production of γ-globulin in multiple myeloma, of insulin in islet cell cancer of the pancreas, of adrenocorticoid hormones in adrenal carcinoma, of gonadotrophin in choriocarcinoma, etc. In the "ectopic" category, one usually points to the production of ACTH, calcitonin, parathormone, gonadotrophin, lactogenic hormones, or anti-diuretic hormone in cases of cancer of the lung, of α-1-fetoglobulin in hepatomas and teratomas, of carcinoembryonic antigen in cancer of the gastrointestinal tract, of Regan isoenzyme in many types of cancer and of muscle aldolase in hepatoma. A special category of phenotypes is the viral tumor antigens.

These findings have proven to be extremely significant in solving problems of the differential diagnosis of cancer and in objectively evaluating the effects of therapy. For example, gonadotrophin production in a non-pregnant female with evidence of tumor points to the possibility of choriocarcinoma, for which specific curative therapy is available. The presence of elevated values for "prostatic" acid phosphatase in a male with urinary symptoms points to a strong possibility of disseminated cancer of the prostate for which specific palliative therapy is indicated. A good therapeutic result is predicted by a rapid fall of the "prostatic" acid phosphatase and recurrence usually by a return of elevated values.

In the case of all of the "expected" phenotype category, the recognition of the tumor product directs the physician's attention to the probable organ site of the tumor.

The measurement of various antigenic materials produced by tumors offers considerable potential for diagnosis as well as serving as an indicator of progression or regression of tumor.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

1. c. (contd.)

The present techniques differ in many ways depending on which polypeptide hormone or carcinoembryonic antigen is being measured. So far, no attempt has been published of the simultaneous determination of several of these phenotypes but rather, the studies deal with single phenotypic proteins. All of these techniques are handicapped by the requirement of large quantities of serum and by long, tedious procedures. The desirability of establishing a cancer cell phenotype profile for individual cancer patients is clear with the use of improved analytical techniques.

At the present time these phenotypes can be classified as follows into several categories.

A. Carcinoembryonic Proteins

- carcinofetal proteins
  - CEA
  - fetoproteins
  - heterophile fetal antigen
  - leukemia-associated antigen
  - fetal sulfoglycoprotein antigen
  - fetal isoenzymes
- carcinoplacental proteins
- placental alkaline phosphatases
  - Regan isoenzyne
  - Nagao isoenzyme
- placental hormones
  - chorionic gonadotrophin
  - placental lactogen
  - plasminogen activators
- tumor angiogenesis factor

B. Non-carcinoembryonic Proteins

- viral antigens
- cell surface antigens
  - TSTA
- non-placental polypeptide hormones
  - ACTH
  - ADH
  - TSH
  - calcitonin
- host proteins in general

Non-carcinoembryonic proteins are being studied in other goals of the phenotype program, e.g., cell surface antigens in Tumor Immunology, TSTA and viral antigens in Viral Oncology, and calcitonin of medullary thyroid carcinoma in Diagnosis and Therapy.

Ovarian Cancer

Ovarian carcinoma appears to be suitable as a prototype for a systematic phenotype study. In a high percentage of cases after surgery, recurrence is manifested by multiple peritoneal implants which produce ascites. The clinical necessity of relieving this ascites results in the collection of ascitic fluid often in volumes of several liters. This fluid usually contains cancer cells, mesothelial cells, lymphocytes, etc. By cloning such mixtures of cells in tissue

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

culture, it should be possible to grow out cancer cells which would be recognizable by a distinctive phenotype. In this regard, ovarian cancer exhibits a high incidence of Regan isoenzyme which provides a highly useful "marker".

This ascites fluid is a resource for cancer-related phenotypes which have not yet been characterized and also provides an indication of the effects of therapy on cancer cells by the measurement of known phenotypes. Thus, in Dr. Fishman's laboratory, the ovarian cancer ascites fluid has been a rich source of the Nagao isoenzyme (a variant of Regan isoenzyme which is L-leucine-sensitive), of transferrins (which Dr. Drysdale is studying) and of histaminase (which is being investigated by Dr. C. W. Lin). Also, Dr. L. L. Stolbach has observed alterations in phenotype during the course of therapy of his patients. The cancer cells have revealed interesting features of mitochondrial membrane alkaline phosphatase when examined by electron microscopy (Dr. M. Sasaki). The organization of this study is the responsibility of W. H. Fishman and L. L. Stolbach.

Other general goals of the Cancer Cell Phenotype Program are defined as "Tumor immunology and cancer-specific antigens" (R. S. Schwartz, L. Nathanson, M. Flax, S. Leskowitz are several of the thirteen investigators involved); "To develop new knowledge on the mechanism of gene expression" (G. Brawerman, C. Sonnenschein and others); and "Viral oncology" (S. Tevethia, R.S. Schwartz, G. Wright).

d. Scientific Staff

The senior staff of Center investigators includes an immunoviral oncologist and a cancer cell biologist.

Coming from Baylor University, Dr. S.S. Tevethia, our immunologist, has joined the staff as of July 1, 1973 as an Associate Professor of Pathology. He is a recognized source of strength in the study of cell surface antigen phenotypes in cells transformed by viruses. Thus, cells transformed by oncogenic viruses acquire tumor-specific transplantation antigen (TSTA) which mediate the development of the cellular immune response of the host leading to the rejection of tumor cells carrying the same antigen. Although immune response to TSTA has been studied in detail, few attempts have been made to understand the basic nature of this antigen or its genetic origin. Tevethia's overall objective is to study the appearance of TSTA during the viral reproductive cycle and the viral abortive cycle and to determine its genetic origin with the use of temperature-sensitive mutants.

Dr. Carlos Sonnenschein, our cancer cell biologist, is a Career Development Awardee of the National Cancer Institute, who has strong interests in cell genetics, including somatic cell hybridization, and in the elaboration by the cell of "estrogen receptor" proteins. These "estrogen receptor" proteins represent phenotypes of a transformed rat uterine endometrial cell line that Sonnenschein has developed.

Both Drs. Tevethia and Sonnenschein's laboratories will be located, as of January 1, 1974, in the TCRC laboratories in the South Cove Building, which will occupy four floors when completed. Both will be alert for opportunities for investigation which the current research proposal might open to them in the future.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Other Tufts scientists who are working in close cooperation with the Director in developing the Cancer Cell Phenotype program are listed below:

Robert S. Schwartz, Professor of Medicine: Immunologic activation of leukemic virus
Larry L. Nathanson, Associate Professor of Medicine: Melanoma, clinical bed unit
Seymour Reichlin, Professor of Medicine: Medullary thyroid carcinoma
Leo L. Stolbach, Associate Professor of Medicine: Chemotherapy
S. Leskowitz, Professor of Pathology: Immunochemistry
G. Brawerman, Professor of Biochemistry: Molecular biology
H. Drysdale, Associate Professor of Biochemistry: Fetoproteins

Staff Recruited for TRC Project

With regard to the specific objectives of grant application, two individuals have been recruited who are not only well qualified, but were attracted by the cancer cell phenotype program over others which had been offered to them. Regarding Dr. C-H. Chang (see attached curriculum vitae), Dr. Florence Moog, Professor of Biology in Washington University, who supervised his Ph.D., writes "He proved to have all the prerequisites of a productive research scientist: imagination, technical versatility, resourcefulness, persistence and boundless energy"... "One thing I can assure you of, however, is that he is exceptionally talented in mastering new techniques, quickly and with great thoroughness". Dr. Sidney Udenfriend, Director of the Roche Institute of Molecular Biology, states "Dr. Chang is a very capable experimental scientist and is highly motivated. This has been a very profitable year for him because he has mastered many new techniques, including radioimmunoassay, tissue culture, isoelectric focussing, affinity chromatography and immunology".
Dr. Chang is joining us as Assistant Professor of Pathology and is expected to assume an increasing important role in the direction and execution of this research program under your consideration.

Dr. Shanthi Raam has just received her Ph.D. in immunology from Dr. F.P. Inman at the University of Georgia. He writes "She is familiar with short-term cultures of murine tumor cells, radiolabeling (^3H) tumor cells proteins, preparation of intracellular fluids, density gradient centrifugation, analytical polyacrylamide gel electrophoresis, preparative block acrylamide gel electrophoresis, liquid scintillation counting and preparation of specific antisera. Dr. Raam is joining us as a post-doctoral research fellow on August 15th and will be working with Dr. Chang and Dr. Fishman to validate the radioimmunoassay techniques for the various carcinoembryonic proteins mentioned in the application.
Two pathologists will be engaged in this research project.

Dr. Lalita Gandbhir is Chief of the Pathology Service at the Pondville Hospital, a Board Member in Anatomic and Clinical Pathology who received much of her training at University Hospital, Boston University School of Medicine. She also engaged in research projects of the Mason Research Institute in toxicologic pathology. She was appointed to Pondville at the same time as Dr. L. L. Stolbach was made Chief of Medicine. She has been collaborating actively

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with him in protocol studies of the Eastern Cooperative Oncology Group and is enthusiastic about the possibility of engaging more deeply in fundamental cancer research based on pathologic material. She has cooperated well with Drs. Stolbach and Fishman in securing tissue specimens from patients with ovarian cancer and other malignancies.

Dr. H. Miyayama is a professionally qualified pathologist who has been trained in the techniques of enzyme histochemistry and of electron microscopy in Professor T. Takeuchi's laboratory in Japan. Professor Takeuchi has had a long professional relationship with Dr. Fishman and sends him his best young pathologists. Dr. Miyayama is located in the TCRC laboratories and would participate with Dr.Gandbhir in working out the best sampling, histologic and cytologic approaches to the evaluation of carcinoembryonic proteins in cells.

Other Faculty who will participate in the project are referred to under Departmental Resources.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

2. Institutional Commitment

a. Physical Plant - Commitments

The University has purchased the South Cove Building which is to house the Tufts Cancer Research Center on half of its eight floors. The first completed floor housing Cancer Enzymology and the Office of Director represents an additional Tufts investment of $120,000. The second, third and fourth floors will require the investment of at least $500,000 of "hard" University funds of which a third has been pledged. Other commitments for support have come from the National Cancer Institute, which has awarded $800,000 for alterations and renovations in 1972 and has approved $150,000 in addition for 1973.

b. Faculty Tenure

At the time of the original TCRC application, Tufts had committed itself to underwriting four tenured professorships, two of which are now in existence (Drs. Fishman and Tevethia). The remaining Professorships will be phased in as expansion of laboratory facilities takes place.

c. Operating Expense

Here the Institutional annual contribution to salaries amounts to $53,000 and to the operation of the present laboratories, $18,000. With the completion of additional two floors in 1973, the operating expense would triple ($54,000).

3. Departmental Resources

a. Pathology

As a Professor of Pathology, Dr. Fishman can obtain, as necessary, the help of his colleagues such as Dr. M. Flax, Chairman of the Pathology Department, and Dr. H. Wolf, Chief of Pathology at the New England Center Hospitals. Both of these men were trained at the Massachusetts General Hospital. These resources are also available to Dr. Gandbhir, the Pondville pathologist who received her training at Boston University Hospital, with Drs. Ira Gore and Max Goodman. In addition, working in Dr. Fishman's laboratory as head of the enzyme histochemistry section is Dr. H. Miyayama, who is a pathologist with excellent training from Dr. Takeuchi's laboratory. He would be working in close cooperation with Dr. Gandbhir in the handling and study of the specimens of bronchial epithelium and bronchogenic carcinoma.

The enzyme histochemical expertise in Dr. Fishman's laboratory is directed towards the fashioning of specific immunochemical techniques using the horseradish peroxidase label along the lines developed by P. Nakane and H. Sternberger for the recognition of polypeptide hormone producing cells of the pituitary gland. Once this capability is achieved, we would be in a position to attempt to demonstrate the various placental and fetal proteins in the cells of the neoplastic and pre-cancerous bronchial epithelium.

b. Medicine

The Medical Oncology component of the Department of Medicine at Tufts is an integral part of the Tufts Cancer Research Center and Dr. Nathanson is to be in charge of an eight bed clinical unit at the New England Center Hospital in addi-

Source: https://www.industrydocuments.ucsf.edu/docs/nfcm0000

tion to operating an active oncology service. Dr. Leo L. Stolbach is Chief of the Medical Service at Pondville Hospital which is an all-cancer state hospital. Dr. J. L. Cohen is Chief of the Oncology Service at the Lemuel Shattuck Hospital. These three clinical investigators are active in the Eastern Cooperative Oncology Group studies evaluating chemotherapeutic regimes for breast cancer, lung cancer, melanoma, ovarian cancer, etc. Dr. Stolbach has been associated with Dr. Fishman since 1956, when he worked in the laboratory as a medical student. At different stages in the development of his career, amongst other pursuits, he collaborated with Dr. Fishman in the study of the isoenzymes of alkaline phosphatase and participated in the discovery of the Regan isoenzyme and in its clinical evaluation. Dr. Stolbach received his training in endocrinology from Dr. Roy Hertz at the National Cancer Institute. He has very good rapport with Dr. Gandbhir and with the administration of the Pondville Hospital.

c. Pondville Hospital

The Pondville Hospital is a 110 bed State hospital in Norfolk (Walpole), Massachusetts, which is devoted entirely to the care of patients with cancer and related diseases. The average occupancy during the past few years has been approximately 76 patients. A modern new hospital complex was completed in 1972. The plans include the most up to date radiotherapy equipment including a betatron and a modern automated clinical laboratory. The present buildings will be turned over to other uses, including conversion to research laboratories and an extended care facility of 50 - 60 beds.

The hospital has an active surgical service with three residents, an oncology trainee and two full-time staff surgeons. In addition, there are a number of visiting surgeons in various surgical sub-specialties.

An active radiotherapy and radiology service is under the supervision of Dr. Ronald Messer and is affiliated with Boston University. Residents and junior staff personnel rotate to Pondville from the University Hospital and Boston City Hospital for radiotherapy and radiology training.

The Pathology Department is under the supervision of Dr. Lalita Gandbhir. The surgical and post-mortem material available affords an excellent opportunity for good clinico-pathological correlation through her staff and consultants. It has a modern autopsy room, surgical pathology laboratory and histology laboratory.

The Department of Medicine in the past was handled on a part-time basis by consulting internists. However, with the appointment of Dr. Leo Stolbach as Chief of Medicine on July 1, 1970, a full-time program for the medical service has been established with the following staff: a junior staff physician, a Tufts Oncology Fellow and two residents.

The patient population in 1972 represented 694 new patients. Since the hospital opened in 1927, a total of more than 55,000 patients have been cared for at Pondville. This affords an excellent opportunity for reviews of various malignant diseases. Hospital summaries are excellent and followup of patients is meticulous.

The medical service participates actively in protocol studies of the Eastern Cooperative Oncology Group (Principal investigator - Dr. Stolbach) and the Cooperative Breast Cancer Group (Principal investigator - Dr. Rita Kelley). This participation affords considerable experience to the house staff in gaining an understanding of carefully designed clinical research studies.

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Daily ward rounds and weekly grand rounds are made on all patients on the medical service. A combined Oncology Conference with the Lemuel Shattuck Hospital has been held on alternate Thursdays at the Pondville Hospital and on alternate Saturdays at the Lemuel Shattuck Hospital. The oncology staff as well as invited speakers conduct these conferences. Also, a weekly Tumor Board Conference is held at the Pondville Hospital to discuss all new patients admitted to the hospital. Members of the pathology, surgery, radiotherapy and medical departments attend this conference, which has been a valuable adjunct to the education of the residents and staff and has resulted in a better coordination of therapeutic efforts. It has been an excellent forum for exchange of ideas between the services at the hospital. Finally, a slide conference is held by the Pathology Department to review interesting patient material on a bi-weekly basis.

Dr. Charles Apffel is devoting full-time to basic research in the area of cancer immunology. His research interests have focused on the regulation of antigenic expression and the role of serum glycoproteins in tumor growth and regression. He has recently been involved in isolation of a protein on the surface of the tumor cell which apparently blocks the immunologic expression of the cell.

Dr. Stolbach's basic research interests have been mainly in the area of serum isoenzyme analysis in patients with malignancy. This work has been carried out in conjunction with the Tufts Cancer Research Laboratory under the directorship of Dr. William H. Fishman. In addition, his laboratory is presently involved in preliminary studies for the measurement of various tumor antigens. Plans are also underway to establish a tissue culture laboratory to grow tumor cells in order to facilitate the study of immunologic factors in host response of cancer patients.

Relationship of proposed research to total research effort of TCRC

The cancer cell phenotype program is at present utilizing ascitic fluids and tumor tissues of patients with ovarian cancer as source materials in the search for carcinoembryonic proteins which could be evaluated in cancer patients in general. We have reached the point where four of these (Regan isoenzyme, HCG, CEA and α-fetoprotein) merit systematic study.

Amongst types of human cancer, bronchogenic cancer would now become the focus of laboratory and clinical interest of the staff of the Center with respect to the relevance of carcinoembryonic proteins from all points of view.

If one thinks in terms of the overall effort, I believe that in the first year, this project could constitute 25% and the percentage of total effort would rise in successive years in proportion to the progress and decisive results obtained. Specifically, this project would establish our immunologic and radioimmunoassay capability in the Cancer Research Center laboratory by supporting Drs. Raam and Chang. (It would also give these young people a research opprtunity which they have merited.) Also, it would establish a productive branch in the Pondville Hospital and would bring to the Pathology Department the opportunity to be engaged in the research program of the Center. Both of these desiderata, if they can be managed, will meet two major handicaps to our progress in research.

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R: REDACTED MATERIAL

CURRICULUM VITAE

WILLIAM H. FISHMAN

Date of Birth: REDACTED

Dependents: REDACTED

Education and Training: B.S. (High Honors in Chemistry), University of Saskatchewan, 1935

Ph.D., Dept. of Biochemistry, University of Toronto, 1939

Post-Doctorate Study, University of Edinburgh, 1940 (with Dr. G.F. Marrian); Cornell University Medical School, 1941 (with Professor V. du Vigneaud)

Professional Societies:

REDACTED

REDACTED

REDACTED

Honors:

1931 - Governor General's Medal and University Entrance Scholarship. Manitoba, Canada

1934 - Univ. Of Saskatchewan Undergraduate Scholarship

1939 - Royal Society of Canada Research Fellow

1940 - Royal Society of Canada Research Fellow

1947 - Federation Fellow, 17th International Physiological Congress, Oxford

1952 - Member, American Board of Clinical Chemists

1950 - Ciba Conference Fellow, London

1957 - Fellow of American Assoc. for the Advancement of Science

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CURRICULUM VITAE - W.H. FISHMAN

1959 - National Science Foundation Travel Award to Japan

1959 - Lecturer, Fifth Glucuronic Acid Research Conference, Tokyo, Japan

1959 - Lecture Tour (Univs. of Tokyo, Nagoya, Osaka, Kobe, Kyoto, Kumamamoto and Kyushu), sponsored by the Japan Biochemical Society, Japan Cancer Society and the Japan Pharmaceutical Society

1962 - Research Career Award of the National Institutes of Health

1964 - Lecturer, Tenth Glucuronic Acid Research Conference, Tokyo, Japan

1966 - Regional Editor for U.S.A. and Canada of "Enzymologia" International Journal of Enzymology, The Hague

1967 - Lecturer in Japan at National Institute of Hygienic Sciences; Tokyo Biochemical Research Institute; Kyushu University, Fac. of Pharmaceutical Sciences; Kumamoto University, Kumamoto; Kobe-Osaka Universities, Kobe; Hakone Conference on Glucuronic Acid, Hakone

1968 - Member, Editorial Board of the Journal of Histochemistry and Cytochemistry, New York

1968 - Listed in "Worlds Whos Who in Science"

1969 - Editor of "Metabolic Conjugation and Metabolic Hydrolysis", Academic Press, A Treatise in 3 Volumes.

1970 - Member, Cell and Tissue Biology Research Evaluation Committee, Veterans Administration, Washington, D.C.

Positions Held:

1936-1939 - Teaching Fellow, Dept. of Biochemistry, Univ. of Toronto, Toronto, Canada

1939-1941 - Post-doctorate research fellowships - Royal Society of Canada

1941-1943 - Instructor of Biochemistry, Bowman-Gray School of Medicine, Winston-Salem, N.C.

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CURRICULUM VITAE - W.H. FISHMAN

1943-1945 - Assistant Professor of Biochemistry, Bowman-Gray School of Medicine, Winston-Salem, N.C.

1945-1948 - Research Associate in Biochemistry (Asst. Prof.) Dept. of Surgery, Univ. of Chicago, Chicago, Illinois

1948-1957 - Associate Director and Chief Biochemist, Cancer Research and Cancer Control Unit, Tufts College Medical School, Boston, Mass.

1956- Consultant in Biochemistry, Holy Ghost Hospital, Boston, Mass.

1950- Special Consultant, Nat. Inst. of Health

1957-1959 - Acting Director and Chief Biochemist, Cancer Research and Cancer Control Unit, Tufts Univ. School of Medicine, Boston, Mass.

1948-1961 - Research Professor of Biochemistry (Cancer), Tufts Univ. Sch. of Med., Boston, Mass.

1956-1962 - Lecturer, Clark Univ., NIH Steroid Training Program

1958- Director of Cancer Research, New England Medical Center Hospitals, Boston, Mass.

1961-1970 - Research Professor of Pathology (Oncology) Tufts Univ. Sch. of Med., Boston, Mass.

1970- Professor of Pathology (Oncology) Tufts Univ. Sch. of Med., Boston, Mass.

1965- Senior Consultant in Oncology to the Lemuel Shattuck Hospital, Boston, Mass.

1966- Consultant Chemist, Boston VA Hospital, Boston, Mass.

1972- Director, Tufts Cancer Research Center

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

R: REDACTED MATERIAL

Name CHANG, CHIN-HAI
(PLEASE TYPE: LAST NAME FIRST)

Mailing Address Dept. of Physiological Chemistry
Roche Institute of Molecular Biology, Nutley
New Jersey Zip Code 07110 Phone No. REDACTED

REDACTED Draft Status Sex Male
REDACTED

SPECIALTIES BY TRAINING OR EXPERIENCE

Protein purification & characterization by zone electrophoresis, gel filtration, ion-exchange chromatography, isofocusing, affinity chromatography, etc; enzyme kinetics; analysis of protein structure; Immunochemistry; culturing of malignant cells; cytokinetics; cell fractionation; molecular embryology; biochemical genetics; Tumor biology; Neurochemistry

ACADEMIC TRAINING

College or University	Location	From Yr.	To Yr.	Major	Minor(s)	Degree
National Taiwan Univ.	Taipei, Taiwan	1961	1965	Zoology	Chemistry	B.S.
Washington Univ.	St. Louis, Missouri	1967	1971	Molecular Biology		Ph.D.

PROFESSIONAL EXPERIENCE (list in reverse chronological order — present position first. If more than five, list last five only)

Employer	From Yr.	To Yr.	Position and nature of duties
Dr. Florence Moog, Biology Dept., Washington Univ., St. Louis, Mo.	1967	1971	Research Assistant
Dr. Sidney Udenfriend, Roche Institute of Molecular Biology, Nutley, N.J.	1971	-----	Postdoctoral Fellow

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PUBLICATIONS (list five most recent)

(1) Chang, C.H. The nature of the heterogeneity of chicken duodenal alkaline phosphatase during post-hatching development. American Zoologist 10: 533 (1970)

(2) Chang, C.H. & Moog, F. Alkaline phosphatase of the chicken duodenum. I. Isolation and partial characterization of the multiple forms of duodenal phosphatase in pre- and post-hatching stages. Biochim. Biophys. Acta 258: 154-165 (1972)

(3) Chang, C.H. & Moog, F. Alkaline phosphatase of the chicken duodenum. II. Enzymatic dissociation of large phosphatase complex predominant in the duodenum before hatching. Biochim. Biophys. Acta 258: 166-177 (1972)

(4) Chang, C.H. & Blume, A. Purification of acetylcholinesterase from cultured mouse neuroblastoma cells by affinity chromatography. (in preparation)

(5) Chang, C.H. & Blume, A. Molecular properties of acetylcholinesterase from cultured neuroblastoma cells. (in preparation)

PROFESSIONAL SOCIETIES AND HONORS

American Association for Advancement of Sciences

[redacted]

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R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: CHIN-HAI CHANG(MR.) DATE OF BIRTH: REDACTED

NATIONALITY: REDACTED VISA STATUS: REDACTED

MARITAL STATUS: REDACTED

CHILDREN: REDACTED

PRESENT ADDRESS: REDACTED

EDUCATION:

R Taiwan Provincial Tainan High School, Tainan Taiwan, Republic of China

R National Taiwan University, Taipei, Taiwan Republic of China; B.S.(Zoology)

1967-1971 Washington University, St. Louis, Missouri, U.S.A. Ph.D.(Molecular Biology)

PROFESSIONAL EXPERIENCE:

1967-1971 Research Assistant; Biology Department, Washington University, St. Louis, Missouri

1971-Date Postdoctoral Fellow; Roche Institute of Molecular Biology, Nutley, N.J. 07110

Ph.D. DISSERTATION:

The nature of the heterogeneity of chicken duodenal alkaline phosphatase during pre- & post-hatching development

Advisors: Dr. Florence Moog, Dr. Oscar Chilson, and Dr. David Kirk

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SPECIFIC TRAINING EXPERIENCE:

Protein purification and characterization by zone electrophoresis, gel filtration, ion-exchange chromatography, sucrose gradient centrifugation, isoelectric focusing, affinity chromatography, Immunoabsorbent, and other supplementary procedures; Enzyme kinetics; Structural analysis of protein; Immunochemistry; Radioimmunoassay; Isotope-labelling studies in cell culture; In vitro culturing of animal cells; Cytokinetics; Cell fractionation; Histochemistry; Hematology

AREAS OF INTEREST:

Enzymology of malignant transformation and metabolic diseases; Developmental biochemistry; Drug metabolism in embryonic development and cell culture; Neurochemistry; Endocrinology; Immunology; Tumor biology; Fertility control

PUBLICATION:

1. Chang, C.H. The nature of the heterogeneity of chicken duodenal alkaline phosphatase during post-hatching development. Amer. Zool. 10: 533, (1970)

2. Chang, C.H. and Moog, F. Alkaline phosphatase of the chicken duodenum. I. Isolation and partial characterization of the multiple forms of duodenal phosphatase in pre- and post-hatching stages. Biochim. Biophys. Acta 258: 154-165 (1972)

3. Chang, C.H. and Moog, F. Alkaline phosphatase of the chicken duodenum. II. Enzymatic dissociation of large phosphatase complex predominent in the duodenum before hatching. Biochim. Biophys. Acta 258: 166-177 (1972)

4. Chang, C.H. and Blume A. Purification and characterization of acetylcholinesterase from cultured mouse neuroblastoma. (in preparation)

5. Stein, S.; Chang, C.H.; Udenfriend, S. and Blume, A. Direct amino acid analysis of the proteins after separation on acrylamide gel at 5 ug level. (in preparation)

REFERENCES:

1. Dr. Florence Moog, Biology Department, Washington University, St. Louis, Mo. 63130, Tel: 314-863-0100 Ext. 4169

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2. Dr. Arthur Blume, Department of Physiological Chemistry, Roche Institute of Molecular Biology, Nutley, N.J. 07110 Tel: 201-235-4717

3. Dr. Sidney Udenfriend, Chairman & Director, Department of Physiological Chemistry, Roche Institute of Molecular Biology, Nutley, N.J. 07110, Tel: 201-235-3731

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BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
George J. Doellgast	Research Fellow	R

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
R	R	☒ Male ☐ Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Fordham College, Bronx, New York	B.S.	1966	Chemistry
Columbia School of Engineering and Applied Sciences	B.S.	1967	Applied Biology
Purdue University, W. Lafayette, Indiana	Ph.D.	1972	Biochemistry

HONORS

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Enzymology	Collaborator

RESEARCH SUPPORT (See instructions)

None

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

Research and Professional Experience:

Ph.D. Thesis Research, 1967-72 — Purdue University, Lafayette, Indiana
Research Assistant, 1964-66 — Columbia College of Physicians and Surgeons and Roosevelt Hospital

Professional Training:

Summers 1964-66, working for Dr. William G. Kelly in the laboratory of Dr. Nicholas P. Christy at Columbia College of Physicians and Surgeons and Roosevelt Hospital, on TLC and liquid-liquid chromatography fractionation of steroids, from synthetic mixtures and biological materials.

Ph.D. research program in the Biochemistry Department of Purdue University; first 1 1/2 years, study of the developing endosperms of normal and high lysine maize in the laboratory of Edwin T. Mertz. Studied changes in dehydrogenase levels, the soluble amino acid pool, and alcohol soluble protein fractions. Remainder of time at Purdue, study of the enzyme α-Isopropylmalate Synthase from Salmonella Typhimurium. Some work on peptide analysis and C^{14}-iodoacetate labeling of sulfhydryl groups, but predominantly worked on improving the purification of the enzyme, using affinity chromatography, purifying a feedback-resistant enzyme by the same procedure, and studying the leucine

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binding site using a number of methods, including affinity chromatography, competition of leucine analogues for the leucine binding site in kinetic assays, and binding of dansyl amino acids as specific fluorescent probes of the leucine binding site.

Techniques Known:

Protein fractionation: ammonium sulfate and polyethylene glycol precipitation; ion exchange, Sephadex, and affinity chromatography; preparative disc gel electrophoresis.
Spectroscopic techniques: ultraviolet, visible and fluorescence spectroscopy.
Counting techniques: liquid scintillation.
Other fractionation techniques: amino acid analysis (Beckmann), liquid-liquid partition chromatography, TLC and adsorption chromatography.
Protein structure: amino terminal analysis (dansylation), tryptic and cyanogen bromide peptide cleavage.

Publications:

Janoski, A.H., Doellgast, G.J. and Kelly, W.G., On the metabolism of 16-α-hydroxy-C-21 steroids. I. Microbial synthesis of radioisotopically labeled 16-α-hydroxylated steroids in high specific activity. Steroids 13, 179, 1969.

Doellgast, G.J. and Kohlhaw, G.B., Affinity chromatography of α-isopropylmalate synthase; evidence for conformational changes. Fed. Proc. 31, 424, 1972. (Abstract)

Doellgast, G.J. and Kohlhaw, G.B., The leucine binding site of α-isopropylmalate synthase from Salmonella Typhimurium. I. Affinity chromatography and reversal of inhibition by analogues. (Manuscript in preparation)

Doellgast, G.J. and Kohlhaw, G.B., The leucine binding site of α-isopropylmalate synthase from Salmonella Typhimurium. II. Dansyl amino acids as specific binding site probes. (Manuscript in preparation)

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CURRICULUM VITAE - LALITA GANDBHIR, M.D.

JUL 25 1973

MEDICAL EDUCATION

June 1957 to April 1962	M.G.M. Medical College, Indore, M.P., India Graduated November 24, 1962

POST GRADUATE TRAINING AND EXPERIENCE

May 1962 to Nov. 1962	M.Y.H. Hospital, Indore, M.P., India Rotating Internship
May 1963 to May 1964	Hamilton General Hospital, Hamilton, Ontario, Canada Anatomic Pathology
June 1964 to June 1965	Women's College Hospital, Toronto, Ontario, Canada Clinical Pathology
Nov. 1965 to Feb. 1967	Mason Research Institute, Worcester, Mass. Staff Pathologist Medical Research, Toxicologic Pathology
March 1967 to June 1968	University Hospital, Boston, Mass. Anatomic Pathology
July 1968 to June 1969	University Hospital, Boston, Mass. Clinical Pathology
July 1969 to Feb. 1970	Assistant Pathologist University Hospital Boston, Mass.
Sept. 1969 to Date	Chief of Pathology Pondville Hospital Walpole, Mass.

LICENSES

Massachusetts and District of Columbia

BOARDS

Anatomic and Clinical Pathology, November 1969.

Memberships

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Personal History

1. Name: MIYAYAMA, Haruhiko

2. Date of birth: REDACTED

3. Place of birth: REDACTED

4. Permanent address: REDACTED
REDACTED

5. Present address: REDACTED

6. Marital status: REDACTED

7. Place of employment: Department of Pathology - School of Medicine, Kumamoto University
Address: 2-1, Honjo 2-chome, Kumamoto City
Position: Assistant

8. Training and work record:

April, 1961 - March, 1963; Premedical course at the School of Medicine - Kumamoto University

April, 1963 - March, 1967; Professional course at the School of Medicine - Kumamoto University (acquired Igakushi which is equivalent to M.D. in the U.S.A.)

April, 1967 - March, 1972: Post-graduate course at the School of Medicine - Kumamoto University (acquired Igakuhakushi which is equivalent to Dr. of Medical Science in the U.S.A.)

March, 1972 - Present; Assistant at the Department of Pathology - School of Medicine - Kumammoto University

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9. Professional Speciality; Medicine, General pathology and enzyme histochemistry

10. Theses and Publication:

	Name of journal/	Date of publication/	Title
1.	Acta Histochem Cytochem Vol. 4, No.2 Page 87-102	June, 1971	Validity of iodine procedure of histochemical method for phosphorylase on skeletal muscle fibers
2.	Acta Histochem Cytochem Vol. 5, No.1 Page 1-17	April, 1972	The light and electron microscopic observations of polyglucose histochemically synthesized in rat skeletal muscles by enzymes, particularly phosphorylase and branching glycosyltransferase
3.	Histochemie 33, Page 31-37	January, 1973	Variation of phosphorylase distribution in skeletal muscles (published by T. Nishiyama and H. Miyayama)
4.	J. Kumamoto Med. Soc. Vol. 45, No.11 (Japanese edition with English abstruct)	November, 1971	Clinicopathological studies on primary brain tumors from the histopathological view points
5.	J. Elet. Micro. (not printed yet)	(1972)	Polyglucose particles synthesized by phosphorylase and branching glycosyltransferase in rat skeletal muscle fibers

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Name Raam, Shanthi
(PLEASE TYPE: LAST NAME FIRST)

Mailing Address Dept. of Microbiology, 831 Bio Science Bldg., University of Georgia, Athens, Georgia

Zip Code 30602 Phone No. 404-542-1434

REDACTED grant

SPECIALTIES BY TRAINING OR EXPERIENCE

Immunology: Special techniques - (1) Sucrose density gradient, (2) Polyacrylamide gel electrophoresis, (3) Molecular Sieve chromatography, (4) Radiolabeling Lymphoid tumor cells, (5) Tissue culture techniques, (6) General Immunological Methods

ACADEMIC TRAINING

College or University	Location	From Yr.	To Yr.	Major	Minor(s)	Degree
University of Ga.	Athens, Georgia	1970	1973	Immunology	--	Ph. D.
Med. College of Ga.	Augusta, Georgia	1968	1970	Virology	--	--
University of Madras	Madras, India	1960	1962	Zoology	--	M. S.
University of Madras	Madras, India	1957	1960	Zoology	Botany	B. S.

PROFESSIONAL EXPERIENCE (list in reverse chronological order — present position first. If more than five, list last five only)

Employer	From Yr.	To Yr.	Position and nature of duties
University of Georgia	1972	Present	Research Assistant
Medical College of Georgia Department of Zoology,	1968	1970	Teaching Assistant
University of Madras, India	1962	1964	Research Assistant

PUBLICATIONS (list five most recent)

(1) Raam, Shanthi and Inman, F. P., Presence of J chains in the intracellular fluid of IgM secreting murine tumor cells. J. of Immunology, 1973 (~~in press~~).
110, 1048

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REFERENCES (give name and business address)

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October, 1972

CURRICULUM VITAE

Personal Data

Name: Leo Lucien Stolbach
Marital Status: REDACTED
Date and Place of Birth REDACTED
Present Home Address:
Present Office Address: Pondville Hospital, Box 111, Walpole, Massachusetts 02081

Professional Data:

Board Certification: Certified by the American Board of Internal Medicine December 8, 1967

Professional Organizations:

REDACTED

Education:

High School: Bronx High School of Science, 1947-1950
Award: Honorable Mention, Westinghouse National Science Talent Search

College: Harvard University, 1950-1954; B.A. cum laude 1954
Major: Biochemical Sciences. Honor's thesis on "The Biosynthesis of Cholesterol"

Medical School: University of Rochester School of Medicine, 1954-1958; M.D. 1958

Positions Held:

Lab Technician, Clinical Pathology Lab., Northern Westchester Hospital, Mt. Kisco, New York; Summer 1952

Lab Technician, Clinical Hematology Lab., Beth Israel Hospital, Brookline, Massachusetts; Summer 1953

Lab Technician, Research Laboratory (with Dr. Ivan D. Frantz, Jr.), Heart Hospital, University of Minnesota Hospitals. Continuation of research work on the biosynthesis of cholesterol, started with Dr. Frantz at the Massachusetts General Hospital for senior honor's thesis in 1953; Summer 1954

Summer Research Scholar of "Aid for Cancer", Boston, Massachusetts. Cancer Research Unit, Tufts University School of Medicine. Research produced simplified technic for measuring prostatic acid phosphatase. Summers of 1955, 1956 and 1957.

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Curriculum Vitae - L. L. Stolbach October, 1971

Summer Fellow, American Cancer Society, University of Rochester School of Medicine. Continuation of research work on prostatic acid phosphatase. Set up method for measuring this enzyme in clinical laboratory at Strong Memorial Hospital, Rochester, New York. Summer 1957

Rotating Intern, University Hospitals, Cleveland, Ohio, 1958-1959

Medical Resident, University Hospitals, Cleveland, Ohio, 1959-1960 and 1963-1964

Clinical Associate, Endocrinology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. First year mainly as a ward physician; cared for patients, predominantly patients with choriocarcinoma and related trophoblastic diseases, adrenal carcinoma, Cushing's syndrome, acromegaly and other endocrine disorders. Second year in research capacity, developed method for extraction and assay of actinomycin D in urine of patients being treated with this drug. 1960-1962

Senior Investigator, Endocrinology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. Research activities in the use of actinomycin D in patients with trophoblastic malignancies were extended. Studies of the hormone relaxin in the serum and urine of patients with trophoblastic malignancy and normal pregnancy. 1962-1963

Research Physician, Lemuel Shattuck Hospital and Instructor in Medicine, Tufts University School of Medicine, July 1964 to June 1966; Senior Instructor in Medicine, June 1966 to July 1968; Assistant Professor of Medicine, July 1968 to present.

Associate Coordinator of Cancer Teaching, Tufts University School of Medicine at the Lemuel Shattuck Hospital, November 1966 to July 1970.

Head of Enzyme Laboratory, Oncology Division, Lemuel Shattuck Hospital, July 1966 to present.

Chief of Medicine, Pondville Hospital, Walpole, Massachusetts, July 1970 to present.

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Publications

William H. Fishman, Ph.D.

1. A Serum Alkaline Phosphatase Isoenzyme of Human Neoplastic Cell Origin. W.H. Fishman, N.R. Inglis, Leo L. Stolbach and M.J. Krant. Cancer Research, 28, 150-154, 1968.

2. New Observations on the Regan Isoenzyme of Alkaline Phosphatase in Cancer Patients. L. Nathanson and W.H. Fishman. Cancer, 27, No. 6, 1388-1397, 1971.

3. The Regan Isoenzyme, A Carcinoplacental Antigen. W.H. Fishman, N.R. Inglis, and S. Green. Cancer Research, 31, 1054-1057, 1971.

4. Carcinoplacental Isoenzyme Antigens. W.H. Fishman. Advances in Enzyme Regulation, ed. by George Weber, Vol. 11, in press.

5. Phenotypes of the Regan Isoenzyme and Identity between the Placental D-Variant and the Nagao Isoenzyme. N.R. Inglis, S. Kirley, L.L. Stolbach, and W.H. Fishman. Cancer Research, in press.

Leo L. Stolbach, M.D.

1. Correlation of Serum L-phenylalanine Sensitive Alkaline Phosphatase, Derived from Intestine, with the ABO Blood Group of Cirrhotics. L.L. Stolbach, M.J. Krant, N.R. Inglis and W.H. Fishman. Gastroenterology, 54, 819-827, May, 1967.

2. Clinical Application of Alkaline Phosphatase Isoenzyme Analysis. L.L. Stolbach. N.Y. Acad. Sci., 166(2), 760-774, 1969.

3. Ectopic Production of an Alkaline Phosphatase Isoenzyme in Patients with Cancer. L.L. Stolbach, M.J. Krant and W.H. Fishman. New Eng. J. Med., 281, 757, 1969.

4. Intestinal Alkaline Phosphatase in Chylous Effusion: Role of ABO Blood Group and Secretor Factor. L.L. Stolbach, M.J. Krant and W.H. Fishman. Enzymologia, 42, 431-438, 1972.

5. Elevation of Total Serum Alkaline Phosphatase, Due to Regan Isoenzyme, in a Patient with Localized Jejunal Lymphoma. L.L. Stolbach, H.H. Skillman and R. Goodman. Arch. Surg., 105, 491-493, 1972.

Chin-Hai Chang, Ph.D.

1. The Nature of the Heterogeneity of Chicken Duodenal Alkaline Phosphatase During Post-Hatching Development. C.H. Chang. Amer. Zool., 10, 533, 1970.

2. Alkaline Phosphatase of the Chicken Duodenum. I. Isolation and Partial Characterization of the Multiple Forms of Duodenal Phosphatase in Pre- and Post-Hatching Stages. C.H. Chang and F. Moog. Biochim. Biophys. Acta, 258, 154-165, 1972.

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3. Alkaline Phosphatase of the Chicken Duodenum. II. Enzymatic Dissociation of Large Phosphatase Complex Predominent in the Duodenum Before Hatching. C.H. Chang and F. Moog. Biochim. Biophys. Acta,258, 166-177, 1972.

4. Purification and Characterization of Acetylcholinesterase From Cultured Mouse Neuroblastoma. C.H. Chang and A. Blume. In preparation.

5. Direct Amino Acid Analysis of the Proteins After Separation on Acrylamide Gel at 5 ug. Level. S. Stein, C.H. Chang, S. Udenfriend and A. Blume. In preparation.

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3. Alkaline Phosphatase of the [illegible] Is Composed [illegible] of Large Phosphatase Complex Predominant in the Duodenum Before Heating, [illegible] Gen. Chem. and Physiol. [illegible]

4. Purification and Characterization [illegible] nd

5. Direct Amino Acid Analysis of [illegible] Gel at pH [illegible] In preparation.

SMOKING QUESTIONNAIRE

NAME_____

ADDRESS_____

TELEPHONE (home)_____(Business)_____

SOCIAL SECURITY NUMBER_____

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1. At present, do you consider yourself primarily a

____a. pipe smoker?

____b. cigar smoker?

____c. cigarette smoker?

____d. non-smoker?

2. During your entire life, have you smoked at least as many as three packages of pipe tobacco? Yes____, No____, Don't know____.

3. How deeply do (did) you usually inhale the smoke? Would you say:
____As deeply into the chest as possible.
____Only partly into the chest.
____As far back as the throat.
____Only into the mouth.
____Just puffed and didn't really draw in.
____Don't know; don't remember.

4. Are(were) there occasions when you smoke(d) more pipefuls than usual? For instance:

____a. at work
____b. when hungry
____c. when doing strenuous work
____d. when playing cards
____e. at weddings
____f. funerals
____g. birthdays
____h. no occasion when you smoke(d) more
____i. other occasion _____ (specify)

5. Have you ever smoked cigars? Yes____. No____.

6. Do you smoke cigars now? Yes____. No____.

7. How many times in the past did you quit smoking cigars then started over again? # of times____. Never____. Don't remember____.

8. About how many cigars do (did) you smoke a day? Answer for each type you smoke(d).

a. Full-sized cigars: #____per day. If not every day: #____per____.

b. Small cigars (cigarillos): #____per day. If not every day: #____ per____.

c. Very small cigars (cigarette size): #____per day. If not every day: #____per____.

9. How much of each cigar do (did) you usually inhale? Would you say:
____inhale(d) almost every puff of each cigar.
____inhale(d) a few puffs of each cigar.
____inhale(d) a few puffs of some cigars.
____do (did) not inhale at all.
____don't know; don't remember

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10. Have you ever smoked any cigarettes? Yes____. No____.

11. Have you smoked at least as many as five packs of cigarettes, that is 100 cigarettes, during your entire life? Yes____. No____.

12. Do you now smoke cigarettes? Yes____. No____.

13. How many cigarettes do (did) you smoke per day? # per day____. If not every day: #____per____.

14. What type of cigarette is (was) it that you smoke(d)?
____a. king size with no filter
____b. king size including regular filter
____c. king size including large filter
____d. king size plus small filter
____e. regular size with no filter
____f. regular size with a filter

15. About how much of each cigarette do (did) you really smoke? Would you say:
____more than 3/4 of each cigarette
____about 3/4 of each cigarette
____about 1/2 of each cigarette
____about 1/4 of each cigarette
____don't know; don't remember

16. How deeply do (did) you usually draw in the smoke? Would you say:
____as deeply into the chest as possible
____as far back as the throat
____as far back as the chest
____only into the mouth
____just puffed and didn't really draw in
____don't know; don't remember

17. How much of each cigarette do (did) you usually inhale? Would you say:
____inhale(d) almost every puff of each cigarette
____inhale(d) a few puffs of each cigarette
____inhale(d) a few puffs of some cigarettes
____do (did) not inhale at all
____don't know; don't remember

18. Do (did) you see yourself as a chain-smoker____, a heavy smoker____, a moderate smoker____, a rather light smoker____, only an occasional smoker____?

19. If you stopped smoking cigarettes, how long ago did you stop? (Specify the number of days, or weeks, or months, or years)_____.
If you have not stopped, check here____.

20. How many times in the past did you quit smoking cigarettes then started over again? # of times____. Don't remember____.

21. The last time you quit (or tried to quit) smoking cigarettes did you weight change:
a. Gained____, Lost____, No change____, Don't remember____.
b. Approximately how many pounds?_____

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22. ANSWER THE FOLLOWING QUESTION IF, AND ONLY IF, YOU HAVE NEVER SMOKED CIGARETTES OR SMOKED LESS THAN 5 PACKS OR 100 CIGARETTES IN YOUR LIFETIME.

From the list below, check all the reasons why you never took up cigarette smoking.

____a.	Family members object	____i.	No sense to it
____b.	Parents didn't smoke	____j.	No appeal or desire
____c.	Religious, moral objection	____k.	Just never started, no reason
____d.	Dirty habit	____l.	Bad habits
____e.	Believe it is bad for health	____m.	Too expensive
____f.	Causes addiction	____n.	Don't know, don't remember
____g.	Dislike smell or taste	____o.	Other_____ (specify)

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From the list below, check all the reasons why you never took up cigarette smoking.

DRINKING QUESTIONNAIRE

NAME_____

ADDRESS_____

TELEPHONE (home)_____(Business)_____

SOCIAL SECURITY NUMBER_____

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1. Do you keep alcoholic beverages in stock at home, including beer and wine? Yes____. No____.

2. Did you ever drink alcoholic beverages in your lifetime, including beer and wine? Yes____. No____.

3. Did you drink at least as many as 10 alcoholic drinks in your lifetime? Yes____. No____.

4. On a long range basis, would you say you generally have been:
   ____a. an occasional drinker
   ____b. a regular drinker
   ____c. once a drinker but now have quit

5. Which of the following statements best describes you at present:
   ____a. a non-drinker
   ____b. an occasional drinker
   ____c. a light drinker
   ____d. a moderate drinker
   ____e. a heavy drinker
   ____f. an alcoholic
   ____g. other _____ (specify)

6. How many times in the past did you quit drinking alcoholic beverages then start over again?
   ____a. none
   ____b. once
   ____c. twice
   ____d. three times
   ____e. more than three
   ____f. don't remember

7. On the line next to each drink listed below, write in the letter of the statement which best identifies how often you drank each five years ago. If you take doubles or triples, count them as 2 or 3 drinks a day, a week, a month, or a year.

   Statement: a. never. b. about one drink a year. c. about one drink a month. d. two or three drinks a month. e. one or two drinks a week. f. three or four drinks a week. g. one drink nearly every day. h. one drink a day. i. two drinks a day. j. three or more drinks a day.

   ____ale
   ____beer
   ____brandy
   ____bourbon
   ____cocktails
   ____cognac
   ____gin
   ____liqueurs (after dinner)
   ____malt liquor
   ____rum
   ____rye
   ____scotch
   ____sherry
   ____vodka
   ____whiskey
   ____wine

8. When drinking, is it (was it) usually at about the same time of day? Yes____. No____. Don't remember____.

9. On the line next to each person you drink (drank) with, write the letter of the statement which best describes how often you drink (drank) with that person.

   Statement: a. never. b. once in a while. c. lessthan half the time. d. more than half the time. e. nearly every time.

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___alone
___with family members
___with friends of the same sex
___with opposite sex friends
___with friends of both sexes
___with acquaintances
___with strangers
___with others _____ (specify)

10. Do (did) you sometimes have a drink too much?
___a. never
___b. seldom
___c. occasionally
___d. frequently
___e. don't remember

11. Have you ever stayed away from work because of a hangover?
___a. never
___b. seldom
___c. occasionally
___d. frequently
___e. don't remember

12. Have you ever had the shakes after heavy drinking?
___a. never
___b. seldom
___c. occasionally
___d. frequently
___e. don't remember

13. On the line next to <u>each</u> reason, write the letter of the statement which best describes how often you drink (drank) for that reason.

Statement: a. never. b. once in a while. c. less than half the time. d. more than half the time. e. nearly every time

___because it tastes good
___to gain self-confidence
___because you find it healthy
___to relax
___to cool off on a hot day
___to take the chill out of a cold day
___to stimulate appetite or digestion
___to relax when watching television
___to help get to sleep
___to help get a business meeting off the ground
___to cheer up when depressed
___to make you feel friendlier

14. Do (did) you drink alcoholic beverages at any of the following times? Next to <u>each</u> time, write in the letter of the statement which best describes how often you drink (drank) at that time of day.

Statement: a. never. b. once in a while. c. less than half the time. d. more than half the time. e. nearly every time.

___ <u>after</u> dinner (supper) when spending the evening out
___ <u>during</u> dinner (supper) when eating out
___ <u>after</u> dinner (supper) when spending the evening at home
___ <u>during</u> dinner (supper) when eating at home
___ <u>between</u> lunch and dinner (supper)
___ <u>during</u> lunch
___ <u>between</u> breakfast and lunch
___ <u>before</u> or <u>during</u> breakfast

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15. Below is a list of reasons for not drinking. On the line next to each reason, write the letter of the statement which best describes how important that reason is or was for your not drinking.

Statement: a. of no importance. b. of little importance. c. of some importance. d. important. e. very important.

I don't drink because:

___family members object
___parents did not drink
___religious, moral objections
___bad habit
___believe it is bad for health
___causes addiction
___dislike the smell
___not socially acceptable
___no sense to it
___no appeal or desire
___just never started, no reason
___too expensive
___tried it but didn't like it
___tried it but got sick
___it was illegal when growing up because of Prohibition
___don't know; don't remember
___other _____ (specify)

16. Please estimate how many ounces of water you drink per day and write the number:_____. (The average drinking glass is about 8 ounces.

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[redacted]

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 17, 1973

Grant Application No. 877R1

To: The committee comprising Drs. Andervont, Gardner, and Huebner

Subject: Herbert B. Herscowitz, Ph.D., Georgetown University, Washington, D. C.
First renewal application No. 877R1
"The Role of the Macrophage in the Immune Response: Effect of Tobacco Products on Macrophage Function."

History

Grant No. 877, for 1972-1973, was awarded in the amount requested ($32,623) with "priority in competition" for two additional years.

Application No. 877R1 requests $29,940. The initial estimate for this year was $26,631.

Documents Submitted (attached)

1. Application date July 15, 1973 (25 pages).

2. Progress Report No. 1, for October 1, 1972-June 1, 1973 (4 pages plus IV tables).

Comment

Except for Dr. Huebner, the composition of the committee for this application has been changed because the misclassification of the proposal last year has been corrected.

Dr. Herscowitz participated in the Macrophage Conference held by CTR late last year.

FWN

F.W.N.

FWN:wg
Encls.

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Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

#877R1

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 13 1973

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal [X] Second Renewal [ ] Date: July 15, 1973

1. Principal Investigator (give title and degrees): Herbert B. Herscowitz, Ph.D.
Assistant Professor of Microbiology
Schools of Medicine & Dentistry
Georgetown University

2. Institution & address: Schools of Medicine & Dentistry
Georgetown University
37th & "O" Streets, N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided: Department of Microbiology

4. Short title of study: "The Role of the Macrophage in the Immune Response: Effect of Tobacco Products on Macrophage Function."

5. Proposed renewal date: October 1, 1973

6. How results to date have changed earlier specific research aims:

At the time of preparation of this application, I can report that the experimental results obtained to date have not altered our original research aims in any way. We have established a working knowledge of our experimental system and are assuredly approaching the point whereby we will be in a position to test the effects of tobacco products on macrophage function.

7. How results to date have changed earlier working hypothesis:

As specified in our original grant application, we felt that we would require at least the first year of this grant support to establish an appropriate in vitro immunological system as a basis for testing our working hypothesis. We feel that we are making adequate progress toward that goal and will soon be in a position to do the appropriate experiments. Therefore, we do not feel that we have to alter our working hypothesis.

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8. Any additional facilities now required? Describe briefly: The second and third phases of our research plan include a study of the effect of tobacco products on macrophage function and their participiation in the immune response. As will become apparent in reading the proposed experiments, we will have to employ a rather crude system for the exposure of cells and whole animals to cigarette smoke. Having had the opportunity to talk to other investigators, I find that such an approach has severe limitations. We feel that these experiments must be carefully controlled as to smoke volume, duration, intensity and frequency; therefore we request the additional funds to purchase a smoke generating device. We realize that there may be certain strictures on available funds and would hope that the Council would provide us with this instrument either as a purchase item or on loan from another investigator not currently using this device.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

A graduate student, Ms. R. DiBlasio, who had been working on a problem related to this application, has completed her requirements for the degree of Master of Science. She has been replaced by Ms. Kyung Jin Kim whose biographical sketch is attached.

10. Append outline of experimental protocol for ensuing year. See attached.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Two manuscripts are in preparation:

DiBlasio, R., Rosenberg, J.B. and Herscowitz, H.B. A hemolytic plaque assay for the detection of 19S and 7S antibody-forming cells to keyhole limpet hemocyanin (KLH). To be submitted to J. Immunol. Methods.

DiBlasio, R. and Herscowitz, H.B. Preparation and properties of a rabbit anti-mouse macrophage serum. To be submitted to J. Immunol.

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12. Summary progress report (append in standard form as separate document, unless recently submitted). See attached.

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R: REDACTED MATERIAL

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Herbert B. Herscowitz, Principal Investigator		REDACTED
Kyung Jin Kim, Graduate Trainee		
Technical		
Jeanette B. Rosenberg		REDACTED
	Sub-Total for A	$14,391
B. Consumable supplies (by major categories)		
1. Disposables and Glassware		1200
2. Tissue Culture Materials		1000
3. Isotopes, Chemicals, Assay Materials		800
4. Animals		1500
5. Miscellaneous		500
	Sub-Total for B	$5,000
C. Other expenses (itemize)		
1. Animal Care		2,000
2. Travel		500
3. Publication		600
4. Equipment Maintenance & Repair		500
	Sub-Total for C	$3,600
	Running Total of A + B + C	$22,991
D. Permanent equipment (itemize)		
1. Smoke Generating Machine		2,500
2. Miscellaneous		1,000
	Sub-Total for D	$3,500
E. Indirect costs (15% of A+B+C)	E	3,449
	Total request	$29,940

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Herbert B. Herscowitz

Signature Herbert B. Herscowitz Date July 15, 1973

Telephone 202 (Area Code) 625 (Number) 7602 (Extension)

Checks payable to

George R. Houston, Jr., Treasurer

Mailing address for checks:

Georgetown University
37th & "O" Streets, N.W.
Washington, D.C. 20007

Responsible officer of institution

Typed Name Mr. Sam A. Kimble

Title Administrator of Sponsored Programs

Signature [signature] Date July 15, 1973

Telephone 202 (Area Code) 625 (Number) 3151 (Extension)

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Justification of Budget

A. Salaries

In accordance with the policy of Georgetown University, I am requesting that R of my salary be paid from this grant. Ms. Jeanette Rosenberg will continue to devote her entire effort toward the experimental goals of this proposal. The values expressed represent an R increase in salary over last year. Fringe benefits have been set at the rate of R by the University.

B. Supplies

These amounts are self-explanatory. Most of our tissue culture will be carried out in disposable lab ware.

C. Other Expenses

An increase in funds is requested in the travel and publication categories over that which was originally requested. The increase in travel is toward the partial cost of attending the Second International Congress of Immunology to be held in Brighton, England during the summer of 1974. I feel that the atmosphere of such a meeting, judging from my experience at the First Congress, will provide up to date information on areas pertinent to this proposal. An increase is also requested for publication costs in view of the fact that we have several manuscripts in preparation.

D. Equipment

An increase in this category is related to the purchase of a smoke generating machine, the justification for which is given in item 8.

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Biographical Sketch

Personal

Name: Kyung Jin Kim
Born:
Citizenship: REDACTED
Marital Status:
Social Security: REDACTED

Education

Sogang University	Seoul, Korea	1969	Biology	B.S.
Louisiana State Univ.	Baton Rouge, La.	1972	Microbiology	M.S.
Georgetown University	Washington, D.C.	--	Microbiology	Ph.D.

Professional Experience

Technical Assistant	Sogang University	1969-1971
Research Assistant	Louisiana State Univ.	1971-1972

Honors

B.S. Degree Cum Laude 1969

Professional Societies

REDACTED

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Research Plan

In view of the possibility that there has been some change in the composition of the Scientific Advisory Board, I will summarily review the background, working hypothesis and goals of our proposed research before outlining the experimental approach to be followed in the ensuing years.

A. Background:

By definition, the basic functional property of a macrophage is its ability to engulf foreign materials. Although they may not demonstrate this capability at all times, the potential for endocytosis is always present. Macrophages take up materials essentially by two closely related processes, phagocytosis (referring to particulate substances) and pinocytosis (usually associated with soluble materials). Both processes are initiated by the interaction of the extracellular foreign material with the cell membrane (1). The particle, after entry into the cell, becomes surrounded by a membrane which eventually closes to form the phagosome. The phagosome detaches and moves to the interior of the cell where it meets and subsequently fuses with a preformed lysosome to form a phagolysosome (2). The hydrolytic enzymes of this secondary lysosome degrade the ingested material and the endocytic function of the macrophage is fulfilled. It is this product of endocytosis which perplexes the immunologist in establishing a functional role of the macrophage in immune induction.

The relevance of phagocytosis to immune processes of host protection is still somewhat uncelar. It has been suggested that there is no correlation between susceptibility to phagocytosis and immunogenicity (3). On the other hand, there is a great deal of evidence which strongly suggests that the macrophage is necessary for the initiation of the immune response (4). Although it was claimed that it is the macrophage that processes the antigen to an immunogenic form and in some cases retains it (5-6), the essentiality of its participation in the induction of the primary immune response was not shown until recently. A requirement for two distinct cell types in the *in vitro* induction of the primary response to SRBC was shown by Mosier (7). In mouse spleen cultures, antibody production appeared to involve both the phagocytosis of antigen by a cell population capable of adhering to glass (macrophage-rich) and antibody production by a non-adherent cell population (lymphocyte rich). Later, this non-adherent cell population was further resolved into its T and B-cell components.

Various methods have been applied toward the separation of lymphoid elements so that studies of cell function could be achieved. Among these methods, was the application of anti-cellular sera to specifically eliminate a single functional cell type. Antisera against partially purified preparations of macrophages have been prepared and tested for immunosuppressive activity (8-10). Unanue (9) found that antimacrophage sera (AMS) caused alterations in both structural and phagocytic characteristics of peritoneal macrophages. Hirsch *et al.* (10) confirmed and extended these findings to show that AMS-treatment resulted in reduced ability of macrophages to adhere to glass and interfered with their ability to phagocytize zymosan particles Further, they showed that treatment of mice with AMS interfered with their ability to resist infection with viral agents, suggesting an altered immune

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mechanism. In extending these experiments to in vivo systems, it was found that pretreatment of mice with AMS resulted in a marked reduction of the primary response to SRBC or diphtheria toxoid (11); however, normal rabbit serum seemed to be as effective in this regard, thereby complicating the interpretation of these experiments. Loewi et al. (12) found that AMS did not inhibit the primary response to SRBC and Unanue (9) failed in an attempt to use AMS to suppress the in vivo response to KLH. On the other hand, it was found that by controlling the dose of immunogen and time of AMS administration the primary response to SRBC could be markedly depressed (13). The work of Jehn et al (14) suggested the necessity of macrophage participation in the secondary immune response. Pierce (15) studied the secondary response to SRBC and suggested that macrophages did not actively participate. On the other hand, Theis and Thorbecke (16) found the SRBC anamnestic response to be macrophage dependent. Using more sophisticated techniques for the preparation and purification of macrophages, Feldmann and Palmer (17) showed that macrophages were required for the induction of the secondary immune response to large complex antigens (i.e. SRBC) but were not involved in the processing of small soluble antigens, suggesting that the requirement for macrophage activity probably depends on the physical size of the antigen. It soon becomes apparent that there is no consistent agreement among investigators as to the requirement for macrophage processing of all antigens to initiate the immune response, however there is compelling evidence that particulate antigens are processed by macrophages for both the primary and secondary responses.

Macrophages also appear to play a role in cell mediated immunity. There is sufficient evidence to suggest that the macrophage functions in the afferent limb of the delayed hypersensitivity response, both by transporting antigen and processing it (18). There have been reports of the successful transfer of tuberculin sensitivity with purified parparations of alveolar macrophages from sensitized animals, suggesting that macrophages can act as effector cells (18). In addition, Thor and Dray (19) have shown that an immunogenic RNA prepared from cells of tuberculin-sensitive donors was capable of transferring delayed reactivity to normal human lymph node cells. There is also evidence to suggest that macrophages contribute to the rejection of allografts. They probably function in the afferent phase of the response by transporting and/or processing antigen. It was found that selective destruction of macrophages or interference with their function prior to grafting resulted in a prolongation of graft survival (20-21). It has also been observed that allografted tumors stimulate an infiltration of macrophages into the tumor area where they contact and kill the tumor cells (22). Further, Old et al. (23) demonstrated that macrophage-rich preparations of peritoneal cells from animals with certain chemically induced tumors could transfer specific tumor immunity to syngeneic recipients. In addition, it has been suggested that non-specific stimulation of macrophages by RES-stimulating agents frequently results in increased resistance to tumors (24).

It is generally accepted that the adaptive immune response evolved as a general vertebrate protective mechanism to eliminate "non-self" components (25). Burnet suggested that the immune system functions as a surveillance mechanism to aid the individual against life-threatening challenge. The surveillance concept has been suggested as a defense in human cancer. In simplest terms, surveillance assumes that tumor cell clones emerge continuously during the

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life of an individual, and that effective mechanisms exist by which the foreigness (tumor clone) is recognized, attacked and destroyed. This can be explained on an immunological basis. There is sufficient evidence to indicate that in the process of transformation to a neoplastic condition, the cell acquires antigens which were not present on the normal parent cell, thus the cell is no longer recognized as self and as a consequence is rendered susceptible to immune attack on the basis of its newly recognized antigenic makeup. One prediction of this theory is that individuals who have immune deficiency, either congenital or acquired, should be subject to a higher incidence of cancer. Indeed, the evidence of Good and Finstad (26) indicates that patients who have congenital immunological defects and patients who have been immunosuppressed following organ transplant develop neoplasms with a greatly increased frequency. Further, in man, the incidence of cancer greatly increases with ensuing age. It has thus been suggested that the development of cancer is associated with a progressive inability to eliminate foreigness which is related to a decrease in efficiency of the immunological defense mechanisms (27). It should be noted that most chemical carcinogens are also immunosuppressive (28) and that many, if not most, viruses known to cause cancer in animals can also suppress the host's immunological reactivity. Thus it would appear that any alteration of the immunological defense mechanism may contribute to the establishment of malignant cells and the eventual demise of the host.

There is sufficient evidence available to indicate that cigarette smoking has an adverse effect on an individual's health. It has been shown that tobacco-derived products are carcinogenic in experimental animals under appropriate conditions. By virtue of their location, alveolar macrophages are of prime importance in protecting the respiratory tract against insult by foreign materials. It is therefore essential that a comprehensive evaluation of the effects of tobacco products on macrophage function be carried out so that we may gain further insight into the effects of smoking on health. However, after a thorough literature search, one finds that there is a paucity of information with regard to the effects of tobacco products on macrophage function.

In this regard, Green and Carolin (29) observed that cigarette smoke depressed the ability of rabbit alveolar macrophages to phagocytize and inactivate S. aureus in an in vitro system. Leuchtenberger and Leuchtenberger (30) compared the effects of charcoal-filtered whole cigarette smoke and unfiltered smoke on the metabolism of mouse and hamster macrophages in vitro. Whereas unfiltered smoke resulted in a series of regressive changes culminating in cell death, the gas phase of charcoal-filtered smoke appeared to enhance metabolic activity as determined by incorporation of ^{3}H-thymidine into DNA. The explanation of this observation is unclear at present, however they suggest that the phagocytic cell may be especially sensitive to the action of cigarette smoke. This is supported by the work of Eichel and Shahrik (31) who reported that charcoal-filtered cigarette smoke inhibited the phagocytic function of human oral leukocytes.

The literature concerning the effect of tobacco products on antibody formation is rather sparse, dated, and confined to in vivo studies. In 1907 Leva (32) studied the effect of injected nicotine on the formation of antibody to the typhus bacillus. In five animals given nicotine (1.5 - 2 mg) daily for

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12-22 days, the immune response was significantly suppressed as compared to the 4 control animals. It should be pointed out that the doses used in these experiments had adverse effects on the animals in that there was convulsion and apathy following nicotine administration. Donzelli (33) acutely exposed immunized animals to cigarette smoke and noted a drop in typhoid agglutinin titer at 6 hours post exposure and which fully recovered in 24-48 hours. In chronically exposed animals, agglutinin titers were reduced to a greater extent than those acutely exposed. Finally in a group of animals immunized during the course of chronic smoke exposure the agglutinin titers were markedly inhibited, the final titer being less than found in unstimulated controls. In another group of experiments, Guardascione (34) found that the injection of nicotine resulted in a reduced response to a heterologous erythrocyte antigen, the decrease being more evident as the dose of nicotine increased. In all of these studies, the effects of tobacco products were evaluated in in vivo systems - one therefore must question whether the immunosuppressive effect observed was due to general protoplasmic poisoning or specific for cells of the immune system.

B. Rationale and Working Hypothesis:

It is postulated that the intact immune system serves as a surveillance mechanism for the inhibition of expression of malignant cell clones. It is apparent that the cells of the reticuloendothelial system are actively involved in maintaining the health of the individual. Numerous studies suggest that macrophages are involved in the induction of both the humoral and cell mediated immune response, probably at the level of antigen processing. There is evidence to indicate that blockade of RES function results in an inhibition of immune responsiveness. Several studies have shown that tobacco products interfere with the phagocytic capabilities of the macrophage. Further, it has been shown that immunosuppression results in an impaired surveillance mechanism so that the patient is rendered more susceptible to insult by pathogenic microorganisms as well as more prone to the development of various forms of malignancy. The question to which we address this research proposal is: Are tobacco products immunosuppressive at the level of the macrophage?

C. Specific Aims:

The specific aims of this research proposal are to study the role of the macrophage in the immune response. We are interested in determining the effects of tobacco products on the ability of macrophages, in general, and alveolar macrophages in particular, to participate in the immune response. Experiments will be devised to clarify and evaluate the following parameters:

(Year 1)

1) To further define the role of the macrophage in the induction of the immune response.

   a) To develop a method for the detection of single antibody forming cells so that we may study cell cooperative effects.
   b) To study the interaction of macrophages with different physical forms of the same immunogen.
   c) To use various methods of cell separation to achieve pure populations of lymphoid cells.

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(1) To prepare and characterize the effects of an anti-macrophage serum on the induction of the immune response.

(Year 2)

2) To determine the effects of tobacco products on the function of macrophages.

a) To study the effect of in vitro and in vivo exposure of peritoneal and alveolar macrophages to nicotine on their phagocytic and other functional capabilities.

(1) To determine the fate and localization of the phagocytized antigen.

b) To study the effect of in vivo and in vitro exposure of peritoneal and alveolar macrophages to cigarette smoke on their phagocytic and other functional capabilities.

(Year 3)

3) To determine the effects of tobacco products on macrophages in relation to their ability to participate in the immune response.

a) To study the effect of these agents on the initiation of both the in vivo and in vitro humoral immune response.

b) To study the effect of these agents on the initiation of the cellular immune response as well as on in vitro correlates of cell-mediated immunity.

D. Experimental Design and Procedures:

1. General Outline

The working hypothesis of this proposal is based on the evidence which suggests that macrophages contribute to the initiation of the immune response at the level of antigen processing and that interference with their phagocytic processes will result in an altered immune response. Although the literature offers numerous references to the participation of macrophages in the immune response, there is considerable controversey as to whether macrophages interact with all forms of antigens in both the primary and secondary immune responses. Therefore, in order to successfully carry out the proposed research regarding the effects of tobacco products on macrophage function, it is first necessary to establish that macrophages are involved in the processing of antigen. Our accomplishments toward that goal as outlined in item 1 of the Specific Aims are given in the attached Progress Report. At the time of this writing, some 3-4 months before the first anniversary of this grant, we feel that we are operating at a pace consistent with the goals set for the first year's accomplishment.

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Once these goals have been achieved, we will study (Year 2) the phagocytic capabilities of macrophages which have been exposed to tobacco products (whole smoke, gas phase and nicotine in vitro as well as the capabilities of macrophages which are derived from animals which have been exposed to tobacco products in vivo. In these studies we will again use the different physical forms of the same immunogen.

In the third phase of this proposal (Year 3) we will study the effects of tobacco products on the ability of macrophages to participate in the initiation of the immune response. Macrophages from tobacco-treated animals or macrophages treated in vitro will be studied for their ability to participate in the initiation of the immune response. Several systems will be employed in these studies. In the first, we will test the ability of tobacco-treated macrophages, after interaction with antigen, to initiate antibody formation in vivo upon transfer of these cells into nonimmune syngeneic animals. In a second system, we will study the ability of tobacco-treated macrophages to initiate the in vitro and in vivo anamnestic responses. And lastly, we will study the effect of tobacco treatment on the ability of macrophages to participate in cell-mediated immune responses.

2. General Procedures:

In view of the fact that our methodological approach has not changed, the procedures to be described in the following sections are essentially the same as submitted in the original application since it was intended to be a three year study. However, there are certain additions to our experimental approach which we feel will add to our basic understanding of macrophage function.

a. Animals and macrophage source:

Two animal systems will be used in these studies. Rabbits will be used as a source of alveolar macrophages for the following reasons: the lung is the prime target of exposure to tobacco products, the rabbit is of sufficient size to yield a workable number of cells, and little is known concerning the contribution of alveolar macrophages to the immune response. The mouse will serve as the source of peritoneal macrophages since a significant amount of data has already been generated concerning the phagocytic capabilities of these cells as well as their participation in the immune response. Although it has been shown that both alveolar and peritoneal macrophages are morphologically similar as to cell type, they appear to exhibit biochemical differences. The hydrolytic enzyme content, which is an important constituent of these phagocytic cells, is 3-4 times greater in alveolar cells than in peritoneal exudate cells (35). The metabolic pathways of the two cell types appear to differ during phagocytosis. Alveolar cells utilize oxidative phosphorylation whereas peritoneal cells appear to utilize the glycolytic pathway for energy (36). In view of these facts we will use both cell types wherever possible so that one will serve as a control for the interpretation of data generated with regard to the other cell type.

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(1) Isolation of Macrophages: [illegible] phagocytic capabilities of macrophages which have been exposed to tobacco [illegible]. The method of Myrvik et al. (37) will be employed to obtain unstimulated rabbit alveolar macrophages. Briefly, rabbits will be sacrificed by injection of a lethal dose of Diabutal (Diamond Labs, Des Moines, Iowa), the chest cavity opened aseptically and the trachea closed by means of a sterile hemostat. The trachea will be transected above the clamp and the lungs, heart and trachea will be dissected out. A volume of 30 ml. of Hank's balanced salts solution will be injected into the trachea until the lungs are distended, the lungs massaged and the fluid drained into sterile centrifuge tubes. The cells will be recovered by centrifugation at 1,500 rpm for 15 minutes and resuspended in culture medium containing 20% normal rabbit serum. The cell suspension so obtained has been shown to be contaminated by less than 2% of other cell types. [illegible] transfer of these cells into [illegible]

Both stimulated and unstimulated peritoneal macrophages will be obtained from mice. For stimulated peritoneal exudate cells, mice will be injected with 3 ml. of fluid thioglycollate medium (Difco Labs, Detroit, Mich)., according to the procedure of Argyris (38). Three days later, the mice will be sacrificed by exposure to chloroform vapor and 3 ml. of BSS containing 30 units of heparin will be injected into the peritoneal cavity. After gentle massage of the peritoneum, the fluid will be collected and cells recovered by centrifugation as above. The cell pellet will be resuspended in culture medium containing 20% of new-born calf serum. Unstimulated peritoneal cells will be collected by a similar procedure except that fluid thiogylcollate will not be injected.

3. Experiments on the Role of the Macrophage in the Immune Response:

a. General Methods

(1) Antigen: [illegible] prior term of exposure to tobacco products, the rabbits of sufficient size

Keyhole limpet hemocyanin (KLH) which is a potent immunogen, inducing high concentrations of antibody, evoking high levels of immunological memory, and involving a large number of cells in the synthesis of antibody (39) will be used as the antigen. KLH obtained as an ammonium sulfate slurry (Pacific Bio-Marine Supply Co., Venice, Calif)., will be purified according to the procedure developed in my laboratory (40). Briefly, after extensive dialysis the material will be concentrated by ultrafiltration using an XM-100 membrane (Amicon Corp., Lexington, Mass). The KLH will be fractionated on a column of DEAE-cellulose employing a linear salt gradient of 0.05 M NaCl -0.05 M PO_4, pH 6.7 to 0.4 M NaCl - 0.05 M PO_4, pH 6.7. The first peak eluted from the column will be concentrated by ultrafiltration, dialyzed and fractionated by molecular sieving on a column of Bio-gel A 5m (Bio-Rad Labs, Richmond, Calif). The first eluted peak, which is the purified KLH, shows a single component as determined by the following criteria: double diffusion in agar, immuno-electrophoresis, analytical ultracentrifugation, and disc electrophoresis. This material will be adjusted to a final concentration of 2 mg/ml, sterilized by Millipore filtration and stored at 4°C until used.

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(1) Isolation of Macrophages

(a) Preparation of dissociated KLH

Purified KLH will be dialyzed against borate-saline buffer, pH 9.0 at 4°C overnight. Since it was found that once dissociated at pH above 8.5, the KLH molecules remain in the dissociated form even after dialysis and extended storage in 0.15 M PBS, pH 7.2 (41); the dissociated KLH will be prepared for use in culture by dialysis against this buffer.

(b) Preparation of aggregated KLH

Purified KLH will be chemically aggregated using the water soluble carbodiimide reagent according to the procedure of Golub et al. (42). In addition, KLH will be rendered particulate by absorption onto bentonite particles (43).

(c) Trace-labelling of KLH

Both associated and dissociated KLH will be trace-labeled with ^{125}Iodine by modification of the chloramine T method as described by Sonada and Schlamowitz (44). We have routinely used this method to obtain labeled proteins of high specific activity.

(2) Tissue culture methods for the initiation of the in vitro immune response

Lymph nodes or spleens from animals previously primed with KLH in vivo will be surgically removed, trimmed of connective tissue and weighed. Cell suspensions will be prepared by disrupting the organ with stainless steel screens. Cells will be washed in 0.15 M PBS, pH 7.2 and adjusted to a final concentration of 2×10^7 nucleated cells/ml. Culture will be carried out in MinimalEssential Medium (Eagle's). The medium will be supplemented with 10% of heat inactivated normal serum (rabbit serum for cultures of rabbit cells and fetal calf serum for mouse cell cultures), 2 mM glutamine, MEM non-essential amino acids, 100 units of penicillin, 50 µg of streptomycin and 1µg of Fungizone as used previously (45). To detect newly synthesized antibody ^{14}C-leucine will be added to the culture medium. Cells will be incubated at 37°C on a roller drum (1 revolution/ 6 min.) in an atmosphere of 5% CO_2 in air.

A portion of the cell suspension will be stimulated with 100 µg of KLH while another portion will be incubated with BSS and will serve as control. After 24 hours, the antigen will be removed from cultures by sedimentation of the cells followed by washing to remove unbound antigen. The culture medium will be changed completely on alternate days. For the determination of newly synthesized antibody, the extra-cellular medium will be dialyzed extensively to remove unincorporated ^{14}C-leucine and subjected to the assay procedure described below. For the determination of cells engaged in antibody formation, cell samples will be removed on various days and assayed by the plaque technique which will be described below.

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(3) Methods for the evaluation of the immune response

(a) Protein and antibody assay

Newly synthesized extracellular proteins will be determined by precipitation of culture fluids with cold 5% TCA as described previously (45). De novo synthesized antibody will be measured by the coprecipitation assay (45) employing ^{14}C-leucine as the precursor of antibody. This method is an extremely sensitive and specific assay of antibody synthesis including antibody of low binding affinity.

(b) Plaque assay

Cells will be assayed for their ability to form hemolytic plaques employing the slide monolayer system of Cunningham and Szenberg (46). Briefly, lymphoid cells at a concentration of 4×10^6 cells/ml. will be mixed with 4×10^8 sheep erythrocytes/ml. which have been coupled with KLH by a modification of the chromic chloride method of Kotoulas and Moroz (47). After the addition of guinea pig complement (1:10), the mixture will be added to each of 3 chambers on a glass slide and incubated for 30 minutes. Plaques will be enumerated by examining the slides under low magnification. To detect indirect plaques (IgG plaques), an appropriate concentration of Anti-IgG serum will be added to the chambers. A full description of the various parameters relating to the establishment of this system as well as kinetic studies employing both rabbit and mouse lymphoid cells are presented in our Progress Report.

b. The Role of the Macrophage in the Immune Response

(1) Preparation of homogenous cell populations

Various procedures are available for the separation and resolution of heterogeneous cell populations into relatively homogeneous cell types. The advantage of using purified cell populations in the study of the cellular interactions in the immune response are obvious if one is to determine the contribution individual cell types to the inductive events of antibody synthesis. Basically, two properties of lymphoid cells have been used to achieve separation, these are based on density differences and the ability of phagocytic cells to adhere to glass.

(a) Separation on the basis of adherence

Cell suspensions will be prepared as described above. The cell suspensions will be incubated in complete MEM at 37°C in plastic petri dishes essentially according to the procedure of Mosier (48). After 2 hours, the supernatant fluid containing non-adherent cells will be removed. The cultures will be incubated overnight and the adherent cell population will be removed with the aid of a rubber policeman.

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(b) Separation on the basis of density

Cells obtained by the described methods will be centrifuged on gradients of bovine serum albumin according to the method of Raidt et al. (49). We will also subject cell populations to density gradient centrifugation on ficoll as described by Gorczynski et al. (50). We have used the latter technique to obtain preparations of macrophages which are 99% pure morphologically.

(c) Treatment of lymphoid cells with carrageenan

Carrageenan, a high molecular weight sulfated polygalactose obtained from marine plants has been shown to be toxic for macrophages both in vitro (51) and in vivo (52). In these studies animals will be injected with varying doses of carrageenan at appropriate times before and after immunization with different physical forms of KLH. Similar experiments will be devised to determine the effect of carrageenan on macrophage function in the in vitro immune responses described herein. These additional studies should further corroborate the functional role of macrophages in antigen processing.

(2) Characteristics of the separated cell populations:

(a) Morphology

The cell populations so obtained will be examined for morphological homogeneity by phase contrast microscopy.

(b) Function

Cells obtained from fractionated gradients will be assayed for functions routinely associated with macrophages. Those fractions determined to have morphological properties of macrophages will be returned to tissue culture and examined for their ability to adhere to plastic. In addition these cells will be incubated with particulate materials and their ability to phagocytize will be evaluated.

(3) Induction of antibody formation

Fractionated cell populations will be tested for their ability to participate in the in vitro immune response. The cell populations will be incubated with the different physical forms of KLH in the presence and absence of macrophages. Cells and culture fluids will be assayed for antibody formation by the methods described. Reconstitution experiments will be performed by adding purified macrophages to the lymphocyte populations followed by in vitro stimulation with KLH. We will also incubate purified macrophages with KLH in vitro and then add these sensitized cells to the lymphocyte population and observe for antibody formation.

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To determine whether previous exposure to KLH by macrophages is required for the induction of the immune response, we will prepare purified alveolar and peritoneal macrophage populations from nonimmune animals or from animals immunized with a different antigen (bovine serum albumin). These macrophages will be added to macrophage-depleted populations of lymphoid cells derived from animals primed with KLH. The magnitude of the observed response should give us an idea as to whether macrophages have a specific function in the antibody response or merely serve as a possible antigen concentrating mechanism in a nonspecific manner.

(4) Macrophage processing of different physical forms of KLH

Purified macrophages will be incubated in suspension culture with ^{125}I-labeled KLH at 37°C according to the procedure of Unanue (53). These experiments will employ the different plysical forms of KLH. The cells will be separated from free antigen, washed and the radioactivity associated with the macrophage will be determined in a gamma spectrometer. To determine the location of the labeled antigen, the macrophages will be disrupted and fractionated by differential centrifugation. To accomplish this, cell extracts will be prepared by disruption of macrophages in a glass Dounce Homogenizer. The homogenate will be centrifuged at 200xg for 5 minutes to remove unbroken cells. The resulting supernatant will be separated into membrane and cytoplasmic constituents by centrifugation. Each of the fractions will be assayed for radioactivity. Such experiments will provide information as to the ability of macrophages to handle the different forms of KLH and will give us some idea as to whether the KLH is processed in the macrophage or if it remains associated with the cell surface. In addition, it will be necessary to determine whether the binding of antigen to macrophages is a function of gamma globulin (cytophilic antibody) or the macrophage surface; to do this we will use heat inactivated serum. Antigen uptake will also be measured in the presence and absence of complement.

(5) Preparation of anti-macrophage serum

An additional approach to the depletion of macrophages from a heterogeneous lymphoid cell population will be through the preparation of a specific anti-macrophage serum. As discussed above, most of these studies performed to date with such a reagent have yielded equivocal results. Macrophages will be obtained from the peritoneal cavity of nonimmune animals as described previously. The cells will be washed and purified by a combination of both of the purification procedures described above. Briefly, cells will be cultured in MEM at 37°C under 5% CO_2. Nonadherent cells will be removed and after a further incubation the adherent cells will be removed and fractionated by density gradient centrifugation on ficoll. Purification will be determined by both morphological and functional criteria. Macrophages so purified will be injected in complete Freund's adjuvant (9) into rabbits by intramuscular and subcutaneous routes. Three weeks later the animals will be given a booster injection of similarly purified macrophages. The animals will be bled at various times and their sera evaluated for specificity and then assayed for antimacrophage activity. Preliminary results of these experiments are in the Progress Report.

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(a) Assay of anti-macrophage activity

Previous reports have shown that serum prepared against mouse macrophages also contained hemagglutinating antibody for mouse red blood cells and showed cytotoxic activity for mouse lymphocytes (9, 13). After assaying our anti-macrophage serum for such activity, appropriate absorptions will be made with mouse RBC and purified suspensions of mouse thymocytes. Finally, we will assay the serum for activity against macrophages by the following criteria: ability to inhibit phagocytic function (9, 14), ability to agglutinate purified macrophage suspensions and ability to injure or kill macrophages in the presence of complement as detected by the release of ^{51}Cr from labeled cells (54). The anti-macrophage serum will then be tested for its effect on the induction of the immune response in two systems. Results of these experiments are in the Progress Report.

(1) In vivo experiments

Both unstimulated KLH-primed mice will be injected with the specific anti-macrophage serum at varying times before and after the injection of antigen in an attempt to achieve macrophage depletion. Control animals will be injected with normal rabbit serum. The animals will be bled at various times thereafter and the sera assayed for circulating antibody levels by passive hemagglutination (55) and by the antigen binding method of Farr (56) as previously described (45).

(2) In vitro experiments

Spleen and lymph node cells from KLH primed animals will be incubated with varying concentrations of specific anti-macrophage serum. The cell population will then be stimulated with KLH and antibody formation determined. Differential cell counts will be made to determine the effective removal of macrophages. Similar cell populations will be treated with normal rabbit serum and serve as controls. In these experiments we will also evaluate the potential handling of the different physical forms of KLH by macrophages.

The experiments described above, constituting the first phase of this proposal, will establish the role of the macrophage in the immune response. Although many of these experiments have been completed and are presented in the Progress Report, the definitive experiments in which we will manipulate the cell population are just at the point where they can be initiated. The above protocols were presented so that new members of the Advisory Board will be familiar with our experimental system.

The remainder of the protocol describes our plans for the next two years.

4. The Effect of Tobacco Products on Macrophage Function

(a) Exposure to tobacco products

In numerous studies attempts were made to simulate actual smoking conditions under experimental situations. However, in evaluating these studies, one must consider that there is tremendous variation among smokers with regard

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to the volume of smoke inhaled with each puff, the number of puffs taken per cigarette as well as the number of cigarettes smoked in a given time period. Therefore, to say that we are simulating actual smoking conditions in experimental situations is somewhat unreal. What we can only hope to do when studying the effects of tobacco products on macrophage function is to use a standardized set of conditions which will be reproducible throughout the experimental design.

We will evaluate the effects of cigarette smoke on macrophage function. In these studies we will use both whole smoke and the gas phase of smoke. Gas phase smoke will be prepared by filtration of whole smoke as described by Leuchtenberger and Leuchtenberger (30). In view of the fact that there are pharmacological differences between components of smoke obtained from the beginning and end of cigarettes, we will use smoke obtained from the middle of a burning cigarette for in vitro studies. Smoke will be collected in a glass syringe fitted with a piece of rubber tubing to hold the cigarette. After determining the volume of smoke obtained from the burning of a whole cigarette, we will use 50 cc of mid-smoke which will be collected in a fresh glass syringe. In order to more rigorously control the variables involved in using such a crude system, we would much prefer to carry-out these experiments with a standardized system such that employing a smoke-generating devise. Therefore, such a machine is requested in our renewal budget.

(1) In vivo exposure to cigarette smoke

Animals from which alveolar macrophages are to be obtained will be exposed to whole smoke or its gas phase for varying periods of time. The animals will be sacrificed and macrophages will be obtained which will be tested for phagocytic function as well as ability to participate in the immune response.

(2) In vitro exposure to cigarette smoke

Macrophages in culture will be exposed to varying volumes of cigarette smoke for varying periods of time according to the procedure of Green and Carolin (29).

(3) Studies with nicotine

In addition to cigarette smoke, we will also study the effect of nicotine on macrophage function. These studies will be included since we can use controlled amounts of a specific component of tobacco. Varying concentrations of nicotine tartrate will be added to cultures of alveolar and peritoneal macrophages according to the procedure of Bond (62).

(b) Experimental Procedures

(1) Effect of tobacco products on phagocytic function

It was observed by Cohn and Ehrenreich (57) that the pinocytosis of certain disaccharides by mouse peritoneal macrophages leads to extensive vacuolization of the macrophage cytoplasm. The vacuoles were shown to be lysosomes containing the pinocytosed disaccharide. Disaccharides which induce vacuolization were

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indigestible and evidently too large to escape readily from the macrophage lysosomes. Thus one has a visible marker to determine the extent of phagocytic function of a cell population.

Pure cultures of alveolar and peritoneal macrophages will be prepared by the methods of adherence and density gradient centrifugation. Cultures will be exposed to smoke and/or nicotine for varying periods of time. Initial experiments will be directed toward the determination of optimal concentration and duration of exposure to tobacco products which will allow for viable macrophage populations. The condition of the cells so treated will be assayed by morphological observation with phase contrast microscopy which will be correlated with cell viability as determined by the trypan blue dye exclusion test. Similar studies will be performed on alveolar macrophages obtained from animals treated with tobacco products in vivo. After exposure to tobacco products, the macrophages will be transferred to fresh media containing KLH bound to bentonite particles and following incubation for varying periods of time, the cells will be fixed in glutaraldehyde and the number of vacuoles in the cytoplasm will be enumerated and compared to untreated controls.

Once that information is obtained, treated macrophage populations will be incubated with ^{125}I-labeled KLH and the uptake, fate and localization of this material will be evaluated as described. In these studies we will employ the various physical forms of KLH. These studies should provide information as to the effect of tobacco products on the phagocytic functions of macrophages.

(2) Comparison of the effects of tobacco-induced immunosuppression with other immunosuppressive agents:

Immunosuppressive agents are widely used in transplant patients as well as in treatment of various diseases. It would be of interest for us to study the effect of immunosuppressive agents (i.e., Imuran, Cytoxan, Methotrexate, etc) on macrophage functions. Experiments will be devised to obtain macrophages from immunosuppressed animals and study their functional capabilities. Similar experiments could be performed on macrophages treated with immunosuppressives in vitro. Such information can be compared with our results obtained by treatment with tobacco products to arrive at a better understanding of the mechanism of action of these agents.

(3) Effect of tobacco products on macrophage participation in the immune response

It has been shown that both free macrophage-processed antigen and antigen bound to macrophage membranes after processing can be immunogenic (58-60) both in vivo and in vitro. The mode of presentation of antigen appears to be a function of the chemical and physical nature of the antigen. The initial phase of this research proposal will provide us with basic information as to the role of different physical forms of antigen in macrophage processing and therefore we can then proceed to study the effect of tobacco products on the immune response. As stated previously, several systems will be used to study these effects; we will study the primary immune response in vivo, the secondary immune response both in vivo and in vitro, and the cellular immune response.

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(a) Primary immune response in vivo:

Both alveolar and peritoneal macrophages will be prepared as described above for in vitro exposure to tobacco products. In one series of experiments, macrophages will be exposed to antigen before they are treated with tobacco products, while in another series, the cells will be treated with the tobacco products before allowed to react with antigen. After various periods of incubation, the macrophages will be collected, washed to remove free antigen, and injected into nonimmune syngeneic animals. Circulating levels of antibody will be measured by the described procedures after collection of serum on various days following primary immunization. Comparison will be made with control animals which will be injected with antigen-treated macrophages which were not exposed to tobacco products. Similar experiments will be carried out with alveolar macrophages obtained from animals exposed to tobacco products in vivo.

(b) Secondary immune response

(1) In vivo

Animals will be injected with a primary dose of KLH in vivo. Macrophages from nonimmune animals will be exposed to tobacco products and antigen as described above and then injected into the primed animals. The levels of circulating antibody will be measured one week after the animals are given the secondary injection. To determine whether macrophage-associated antigen can prime animals for a secondary response, we will inject macrophages incubated with antigen either with or without treatment by tobacco products and then challenge with free KLH. Serum will again be collected and assayed for antibody levels.

(2) In vitro

Lymph nodes and spleens from primed animals will be removed and macrophages will be separated from the population as described. These macrophages will be treated with tobacco products and antigen and then added back to the lymphocyte population. Antibody formation will be determined by the coprecipitation method and by plaque assay. In addition we will determine the effect of tobacco products on the ability of purified preparation of alveolar macrophages from nonimmune animals to present antigen to primed lymphocytes in this system.

(c) Cell-mediated immunity

Unanue and Feldman (61) have shown that in injection of macrophage-bound antigen can induce a state of delayed sensitivity in animals. Alveolar and peritoneal macrophages will be harvested from nonimmune animals and incubated with KLH. After washing, the antigen-containing macrophages will be injected alone or in Freund's complete adjuvant into the foot pad. Two to four weeks later the animal will be challenged with soluble antigen injected into the skin. The degree of erythema and induration will be measured some 24-48 hours later. These experiments will then be repeated with macrophages fron animals exposed to tobacco products in vivo as well as with macrophages treated in vitro.

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Thus, the above studies should provide us with some clear cut information concerning the role of tobacco products on macrophage function.

E. Significance of the Proposed Research

It is well appreciated that the normal functions of the immune system protect the indivudal from constant challenge by foreign materials. A breakdown of immine function, whether under natural circumstances (immunological deficiency disease and aging) or intentionally induced (treatment with immunosuppressive agents) renders the individual subject to infection by microorganisms and prone to the development of various forms of cancer.

A potentially essential link in the chain of events leading to the initiation of both the humoral and cellular immune response is the interaction of antigen with macrophages. It is suggested that this interaction results in the processing of antigen thus rendering it immunogenic. Treatment of macrophages with various agents known to interfere with their phagocytic function results in a suppressed immune response. The proposed research should generate information which may provide insight into the possible means by which tobacco products may affect macrophage function. To date, there have been no systematic approaches to the study of the effect of tobacco products on macrophages with respect to their participation in the immune response. Since alveolar macrophages play an important role in the maintenance of lung stability, the proposed research is of direct significance to the understanding of the relationship between tobacco smoke and chronic lung disease.

In addition, through the proposed research, we hope to elucidate the mechanism of antigen recognition. If we can determine which physical forms of antigen are processed by macrophages, we may acquire a better understanding of why certain antigens lead to a breakdown of immune surveillance (immunological tolerance) whereas others initiate the immune response.

[illegible] lymph nodes and spleens from primed animals will be removed and macrophages [illegible]

[illegible]

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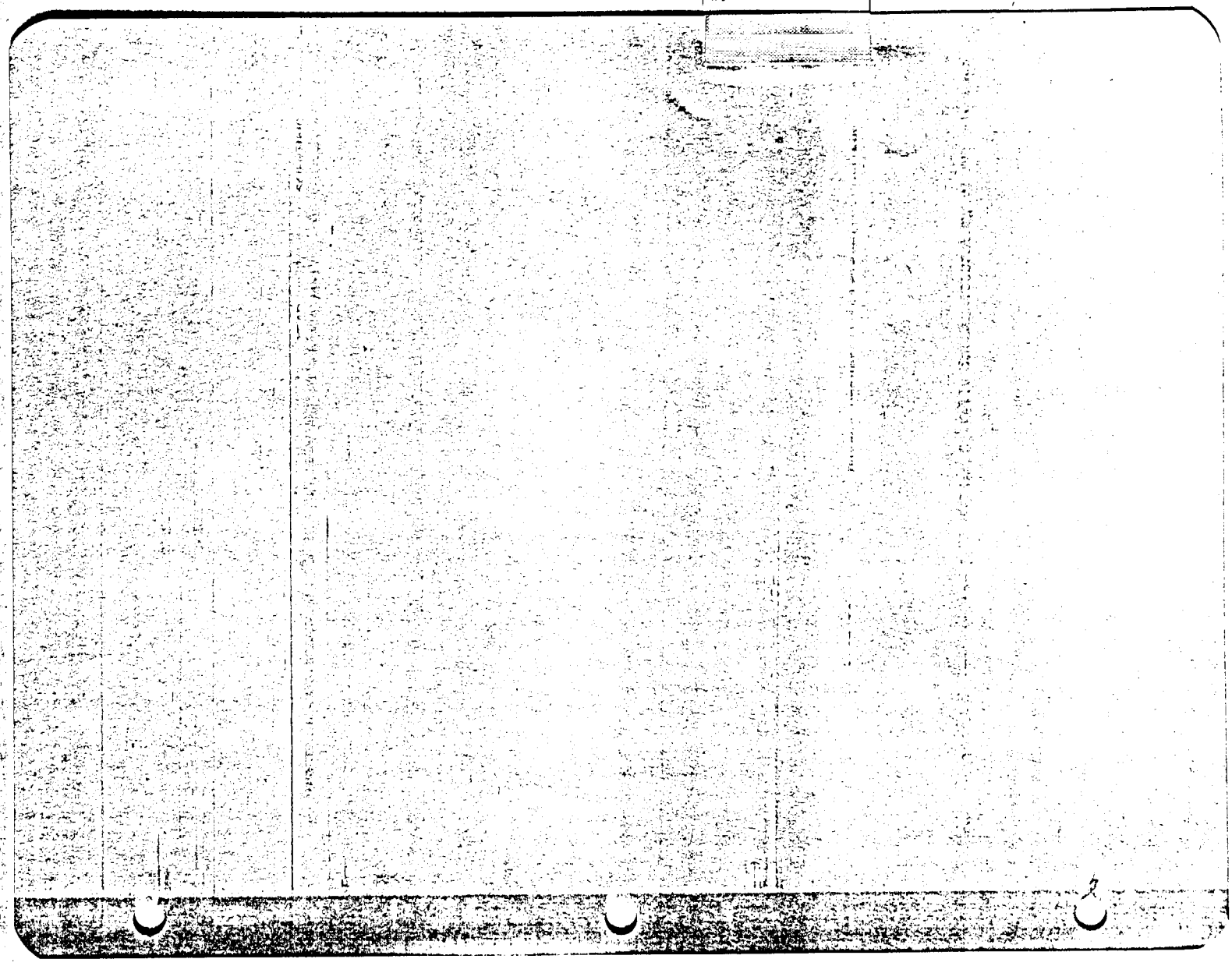

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 26, 1973

Grant application #924
CANCER

TO: The committee comprising Drs. Andervont, Gardner and Huebner

SUBJECT: John C. Houck, Ph.D., Childrens Hospital, Washington, D.C.
New application #924
"Search for Lung Chalone"

History

This proposal was case #201, and formal application was encouraged.

Application #924 requests $36,098. A possible request for two additional years is stated, to be contingent upon observations made during the first year.

Documents Submitted

Attached is letter dated July 23, 1973 with undated application received by us July 26, 1973.

Reprints of references 1, 5, 7, 8 and 9, listed on page 2C of the application have been provided, and will be forwarded if you so request.

Comment

Staff comment may follow in due course.

FWN

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Children's Hospital Research Foundation

2125 13TH STREET, N.W.
WASHINGTON, D.C. 20009

ROBERT H. PARROTT, M.D.
DIRECTOR

JOHN C. HOUCK, PH.D.
SCIENTIFIC DIRECTOR

ROBERT V. MORIN
EXECUTIVE DIRECTOR

July 23, 1973

Robert C. Hockett, Ph.D.
Acting Scientific Director
The Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

Enclosed please find two copies of our application for research support to permit us to search for, identify, and isolate a lung chalone. As indicated in this document, if we are unable to demonstrate chalone-like activity in extracts of lung used against human lung cancer cells in culture, we would immediately stop wasting our time and terminate the grant. I anticipate that we would have a "yea or nay" answer to this critical question by the end of the first year. If on the other hand such "chalone-like" inhibitory activity can be shown, we would expect to pursue the isolation, purification, and characterization of this active material during the following two years of requested funding.

Thanking you for your consideration in this matter, I am,

Sincerely yours,

John C. Houck

John C. Houck, Ph.D.
Scientific Director
Research Foundation
Children's Hospital

Professor of Child Health
and Welfare
George Washington University
Medical School

JCH/jr
Enclosures: 2

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#924

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 26 1973

Application for Research Grant
(Use extra pages as needed)

Date:

1. Principal Investigator (give title and degrees):

John Candee Houck, Ph.D. — Scientific Director, Research Foundation, Children's Hospital, Washington, D. C. — Professor of Child Health and Development, George Washington University Medical School

2. Institution & address:

Children's Hospital Research Foundation
2125 13th Street, N. W.
Washington, D. C. 20009

3. Department(s) where research will be done or collaboration provided:

Biochemical Research Laboratory

4. Short title of study:

Search for Lung Chalone

5. Proposed starting date: January 1 1974

6. Estimated time to complete: Three Years

7. Brief description of specific research aims:

(a) To isolate and establish in vitro pure cell lines of human squamous epithelial and "oat cell" carcinomas from resected lung tumors.

(b) To determine quantitatively the rate of proliferation of both of these cell lines in vitro from H^3 thymidine incorporation studies and by calculating their population doubling times.

(c) Using these two cell systems, the effects of lung tissue extracts upon their mitotic activity in vitro will be determined.

(d) Mitotic inhibitory activity for lung carcinoma cells in vitro from aqueous extracts of normal cadaver or animal lung will be characterized in terms of cell and tissue specificity, cytotoxicity, and chemistry (size, isoelectric point).

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8. Brief statement of working hypothesis:

It has been proposed by Bullough and Laurence that most cells are under the control of a specific and endogenous negative feedback inhibitor of mitosis called "chalone". Chalones have been defined operationally for epidermis, fibroblast, lymphocyte, melanocyte, and a number of other cell types as indicated in the enclosed review reprint. Our hypothesis is that lung, too, is under the control of chalone, as has originally been suggested in a small paper by Simnett, Fisher, and Hepplesten, entitled "Tissue-specific inhibition of lung alveolar cell mitosis in organ culture" (Nature 223: 944, 1969). All chalones that have been described to date also specifically inhibit the mitotic activity of the appropriate tumor cell! Tumor cells require more chalone than do normal cells for an equal degree of inhibition. Chalone appears to inhibit both normal and tumor cells in the G_1 phase of the mitotic cell cycle. Therefor by adding lung chalone (if such exists) in vivo, all lung epithelial cell proliferation will cease; when the dose of chalone is reduced after 1 or 2 days, all the tumor cells will escape chalone control and enter in a synchronized fashion the "S" phase of the cell cycle, during which time they will be extremely vulnerable to chemotherapy by DNA poisons. The normal cells will still be inhibited by chalone in G_1 because they bind the chalone better than do the tumor cells and hence require 2-3 times less chalone for inhibition. By using lung chalone to specifically synchronize tumor cells in vivo, a profound increase in the efficacy of chemotherapy of pulmonary carcinoma might result. This possibility strongly directs us to search for and purify and characterize a chalone for lung.

9. Details of experimental design and procedures (append extra pages as necessary)

Specific Aim #1 is to establish in vitro cell lines of human pulmonary squamous epithelial carcinoma and "oat cell" carcinoma.

Both Georgetown University Hospital (Dr. John Potter) and the Washington Hospital Center are sister institutions of Children's Hospital in this city. At both these institutions, a number of lung resections are done every week. With the cooperation of the Departments of Pathology and Surgery at both of these institutions, we will be able to obtain tissue samples from lung resections which have been diagnosed pathologically to be pulmonary squamous epithelial carcinoma or "oat cell" carcinoma. The tumor samples will be introduced into three media: (1) Medium 199 (a complete general medium) containing 20% serum, (2) L-15 containing 20% serum which is a medium uniquely poor in glucose, highly rich in amino acids, and particularly good for tumor cells, and (3) McCoy's Medium containing 20% serum, which has 4 X as much glucose as does Medium 199 and is also helpful for growing certain kinds of tumor cells. All these media will be supplemented as needed with Pen-Strep and Fungizone to limit the bacterial and fungal contaminant growth in vitro of these explants. After 10 days to 2 weeks, a large number of cells will start to proliferate from the explant onto the glass wall of the incubation vessels. At this point, the serum concentration in the medium will be reduced to 10% and 1 mg/ml of fibroblast chalone (1) will be introduced into the vessels. Preliminary evidence indicates that the mitotic activity of the fibroblasts from lung tissue explant will be reduced by over 90% at this concentration of chalone (1). In this fashion, we would hope that within a few weeks we will have cultivated pure lung carcinoma cells, free of both microbial, fungal, and fibroblast contamination.

These cells, which adhere to glass coverslips in Leighton tubes, will be harvested at various periods of time after seeding and their histology will be determined both at the light microscope and electron microscopic levels.

Specific Aim #2 is to determine proliferative rates in vitro.

The proliferative rate of these two human lung tumor cell types in vitro will be determined by two different techniques:

(a) By determining the rate of incorporation of tritiated thymidine into macromolecular DNA in a manner analogous to that described by us for fibroblasts in vitro (1). Thus cultures at various days after seeding a known number of cells (determined in a hemocytometer) into replicate Leighton tubes will be incubated for various periods of time (ranging from 1 to 24 hrs) with a single pulse of 1 µci of H^3 thymidine. At the conclusion of the incubation,period, these cells will be rinsed with ice cold isotonic saline to remove free isotope, serum, and medium, and then precipitated in the Leighton tube with 5% trichloracetic acid. A rubber policeman will be used to collect all this precipitate,

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which will be washed twice with TCA and then solubilized in the usual fashion using an NBZ solubilizer. The total uptake of H^3 thymidine into macromolecular DNA will be determined by liquid scintillation counting in the usual fashion. From these data, the rate of thymidine incorporation by these cells during cultivation may be quantitatively compared with their cell number and the age of the culture.

(b) Proliferative rates of these cells may also be determined, since they adhere to glass, in the manner developed by Raff and Houck (2). A known number of cells are seeded into replicate Leighton tubes and at various periods of time after this inoculation of cultured cells, the numbers of cells are counted, using a Whipple eyepiece and an inverted microscope. In this fashion, non-destructive analysis of the number of cells in each culture may be obtained daily or twice daily for a week until the cells are so dense as to defy differentiation by the Whipple eyepiece. A plot of the log of the number of cells in these cultures versus time will give an initial straight line, the slope of which will permit the calculation of the time required for the population of cells to double. This population doubling time represents a quantitative measure of the proliferative rate of cells cultivated in vitro (2).

In this fashion then, we will have two measures of the proliferative rate of human lung carcinoma cells in culture in order to explore the possibility that the lung cells are under chalone control.

Specific Aim #3 is to demonstrate the mitotic inhibition by pulmonary tissue extracts.

Using the in vitro-cultivated lung tumor cells described above and measuring the two parameters of proliferation indicated above, extracts of animal and cadaver pulmonary tissue (from the Department of Pathology at Children's Hospital) will be obtained via the extraction of macerated lung tissue into 10 ml of 0.15M NaCl in the cold, using an Omni Mixer as has been described by us for other tissues (3, 4). This extract will be allowed t o stand overnight in the cold in order to swell and thereby permit molecular diffusion. Subsequently, the tissue brei will be centrifuged in the cold and the clear supernatant removed and subjected to Amicon Daiflo ultrafiltration, using a membrane which will pass molecules of 500 daltons or less. In this fashion, large volumes of pure water may be used to "wash" the extract, removing thereby both salt and small molecular weight materials such as cold thymidine which might, by diluting the pool size of the H^3 labeled thymidine, complicate the measurements of the proliferative rates of the cells when using the thymidine uptake technique. After seven volumes of clear water have been washed through the extract in the Amicon Diaflo filtration system, the extract will be concentrated by using nitrogen pressure to express a large amount of water through the filter from the extract. This concentrated material will then be lyophilized.

Various concentrations of lyophilized material will be made up in the appropriate medium and added to the cultures of lung tumor-derived cell lines mentioned above. The effects of these extracts upon the cell proliferation rate of both cell types will be determined quantitatively. The results of this experiment will dictate the subsequent steps: (a) if there is no apparent inhibition by this technique and careful repetition confirms this, at this point the project is over; (b) if on the other hand, there is evidence of inhibition of mitosis from both techniques, then further characterization and purification of the inhibitory principle in this tissue extract seems mandatory.

Specific Aim #4 is to characterize the mitotic inhibitory activity of pulmonary tissue extracts.

A number of steps will be involved in the characterization of the apparent mitotic inhibition, namely (a) biological, and (b) chemical. The biological studies will be addressed to the question of the specificity of this apparent mitotic inhibition of pulmonary carcinoma cells in culture. Specifically, various concentrations of lung extract described above which have been shown to inhibit the proliferative rate of these pulmonary tumor cells in vitro will be tested upon other cell types available in this

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laboratory: diploid human fibroblasts, human lymphocytes which have been stimulated to transform via either lectins or antigens, HeLa cells, colon carcinoma cells, and human choriocarcinoma cells. If the specificity of the chalone concept is borne out for lung tissue, this lung extract, while it might be contaminated with fibroblast chalone, should in fact have no effect on human colon, chorion, lymphocyte, or HeLa cells in vitro. Extracts of tissues other than lung, such as muscle, kidney, liver, will be tested at the same concentration as the lung extract upon the cultured cells of pulmonary carcinoma as described above in an attempt to demonstrate that only extracts of lung tissue have material in them capable of inhibiting the proliferation of these cells.

Finally, the question of cytotoxicity is enormously important in the search for chalone, namely, according to Houck's Law(5), "dead cells don't divide," and the corollary of this is that "dying cells divide damn slowly" (see enclosed review reprints). Our primary measures for studying the viability of cells cultivated in vitro is firstly by determining their ability to exclude a vital dye, secondly by the reversibility of the mitotic inhibition subsequent to the removal of the extract being tested from the culture, and finally by determining whether any inhibition of initial rates of incorporation into the cells of C^{14} labeled amino acids into macromolecular protein has occurred. Cytotoxic agents usually are found to inhibit this protein synthetic rate very quickly. However, with specific DNA synthesis inhibition, usually after some time there is a marked inhibition of the rate of protein synthesis by the inhibited cell. Therefore only the effects of chalone upon the initial rates of protein synthesis will be terribly helpful in making a judgment to include or exclude cytotoxicity as a mechanism of the mitotic inhibition of lung tissue extracts upon these cells.

The initial chemical characterization of this mitotic inhibitory activity will be through sequential fractionation on the basis of molecular size, using Amicon Diaflo filters in series (6). In this fashion, we can accumulate large amounts of the crude extracts on the basis of molecular weights into weight ranges above 300,000 daltons; between 300,000 and 100,000 daltons; between 100,000 and 50,000 daltons; between 50,000 and 30,000 daltons; between 30,000 and 10,000 daltons; and finally between 10,000 and 1,000 daltons. This type of molecular sieving has been done by us for fibroblast mitogen (7), fibroblast chalone (1), and lymphocyte chalone (8, 9). All of these molecular weight ranges can be tested for their cytotoxic-free inhibition of the proliferation rate of pulmonary carcinoma cells in vitro as described above. Hopefully only one of these molecular weight ranges will contain cytotoxic-free mitotic inhibitory activity. This molecular weight range material will then be subjected to isoelectric focusing (10) after determining that the mitotic activity is stable upon standing in high concentrations of sucrose for 2-3 days at various pH (7). Isoelectric focusing will firstly demonstrate the isoelectric point of the material and secondly provide an enormously large purification of this material.

Specific Aim #5 is to isolate, purify, and chemically characterize this material.

Using in sequence molecular sieving by ultrafiltration and isoelectric focusing, the subsequent partially purified inhibitor of the proliferation of pulmonary carcinoma cells in vitro will be subjected to preparative acrylamide gel electrophoresis (11) and the active electrophoretic fraction will be determined as described above by studies of the inhibition of the proliferative rates of these pulmonary carcinoma cells. The active material from this step will be subjected to analytical acrylamide gel electrophoresis at three different pH and in the presence of sodium dodecyl sulfate (7). If the material is not homogeneous, at this point in the purification scheme as judged by these rigorous criteria, then further purification steps will be employed, namely column chromatography, using DEAE cellulose at slightly alkaline pH and a salt gradient, or CM cellulose at pH 4.5 using a salt gradient. Again, the active fraction will be determined biologically as described above, and its homogeneity will be determined by acrylamide gel electrophoresis

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at three different pH and SDS electrophoresis. At some point during this process, essential homogeneous material should result. At this time, the molecular weight of this material will be determined firstly by its elution from columns of P200 or P100 or P50 or P10, depending on molecular weight (12), and secondly by migration in SDS electrophoresis compared with appropriate standards (13). In this fashion then, on the basis of both exclusion volume and charge density, we will be able to determine with some precision the molecular weight of the chalone (7).

Finally, the carbohydrate content and amino acid content of the purified material will be determined by the use of appropriate chemical and chromatographic techniques as well as automatic amino acid analysis (7).

In summary, all chalones that have been demonstrated to date, namely epidermis, fibroblast, melanocyte, and lymphocyte chalones, are all capable of specifically arresting the mitotic activity of tumor cells of that cell type. Our data, which is probably best defined in the lymphocyte and fibroblast systems, also suggests that while the tumor cell will respond to the chalone, it will not in fact quantitatively be as effective against tumor cells as it is against normal parent cell type. Thus, doses of approximately 3 X larger than normal are required to give equivalent mitotic inhibition for the tumor variety of cells.

Our proposition is that if there is a lung chalone, then one could attempt to use the chalone as an adjunct to chemotherapy on the basis of the following reasons: (a) because of chalone cell specificity, patients receiving amounts of chalone sufficiently large to inhibit the proliferation of lung carcinoma cells in vitro, regardless of their location (such as metastisized cells), would have an inhibition of proliferation of only lung cells for a finite period of time; (b) as the concentration of chalone after a few days was reduced, the tumor cells would escape from chalone control and enter the S phase portion of the mitotic cell cycle. (This proceeds from the fact that most of the chalones we know about inhibit in G_1.); (c) As these tumor cells escape from chalone control and enter S phase in a synchronized fashion, they become enormously vulnerable to those chemotherapeutic agents which function by destroying cells making DNA. (d) further, while tumor cells are entering in a synchronized manner into "S" phase, the normal parent cells of this tumor type will still be under chalone control and hence not entering the "S" phase portion of the mitotic cell cycle. Therefore the normal cells would be largely resistant to chemotherapeutic toxicity and thus the efficiency of chemotherapy would be considerably improved.

It is for this ultimate therapeutic purpose that we seek to search for and purify a lung chalone.

References

1. Houck, et al., Nature New Biol, 240: 210 (1972).
2. Raff and Houck, J. Cell. Physiol., 74: 235 (1969).
3. Houck and Jacob, Proc. Soc. Exp. Biol. Med., 105: 325 (1960).
4. Houck, Ann. N. Y. Acad. Sci., 105: 767 (1963).
5. Houck and Hennings, Fed. Europ. Biochem. Soc Letters, 32: 1 (1973).
6. Zipilivan, et al., Anal. Biochem., 30: 91 (1969).
7. Houck and Cheng, J. Cell. Physiol., 81, 257 (1973).
8. Houck et al., Science, 173:1139 (1971).
9. Houck et al., Clin. Proc. Children's Hospital, 28: 130 (1972).
10. Vesterberg and Swenson, Acta Chem. Scand., 20: 820 (1966).
11. Williams and Reisfeld, Ann. N. Y. Acad. Sci., 121:373 (1964).
12. Piez, Anal. Biochem., 26: 305 (1968).
13. Weber and Osborn, J. Biol. Chem., 244: 4406 (1969).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Complete cell culture facilities are available as well as a liquid scintillation counter, an isoelectric focusing preparative device, preparative and analytical acrylamide gel electrophoresis device, a cold room, refrigerated centrifuges, refrigerated fraction collectors for column chromatography, automatic amino acid analysis facilities, and the necessary facilities for histology and histochemistry.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

Complete cell culture facilities are available as well as a liquid scintillation counter, an isoelectric focusing preparative device, preparative and analytical acrylamide gel electrophoresis devices, a cold room, refrigerated [illegible] for column chromatography, automatic amino acid analysis system, the necessary facilities

Biographical Sketch

Principal Investigator

Name: John C. Houck

Title: Scientific Director
Research Foundation
Children's Hospital of D. C.

Date & Place of Birth: REDACTED

Education: B.A., 1953
Columbia College, New York

M.S., 1955
University of Western Ontario, Canada

Ph.D., 1956
University of Western Ontario, Canada

Awards: National Research Council (Canada)
Research Fellowships, 1953-1956

New York Academy of Science
A. Cressy Morrison Award, 1962

American Dermatological Association
Research Award, 1965

Societies: REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

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Biographical Sketch, John C. Houck, cont'd

Professional Experience:	1969 to Present	Scientific Director Research Foundation Children's Hospital Washington, D. C.
	1969 to Present	Professor of Child Health and Development George Washington University Medical School
	1967-1969	Associate Professor Departments of Pediatrics and Biochemistry George Washington University Medical School
	1967 to Present	Professorial Lecturer Department of Biochemistry Georgetown University School of Medicine
	1960-1967	Associate Professor Department of Biochemistry Georgetown University School of Medicine
	1958-1960	Assistant Professor Department of Biochemistry Georgetown University School of Medicine
	1959 to Present	Director Biochemical Research Laboratory Children's Hospital of D. C.
	1958-1960	Director, Surgical Research Laboratory and Metabolic Laboratory Georgetown University School of Medicine
	1957-1958	Senior Research Fellow Surgical Research Laboratory Georgetown University
	1967 to Present	Editor-in-Chief, INFLO Newsletter of the International Inflammation Club

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R: REDACTED MATERIAL

Biographical Sketch

Research Associate

Name:	Richard F. Cheng
Title:	Research Associate Biochemistry Research Laboratory Children's Hospital of D. C.
Date & Place of Birth:	REDACTED
Education:	D.D.S., 1966 National Taiwan University
	Ph.D., 1972 George Washington University

Professional Experience:

1969 to Present	Research Associate Biochemical Research Laboratory Children's Hospital of D. C.
1968-1969	Research Assistant Department of Biochemistry George Washington University
1967-1968	Dental Resident National Taiwan Univ Hospital
1966-1967	Dental Officer Dental Corps, Chinese Navy

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Publications

1. Houck, J. C., Irausquin, H., and Leikin, S., Lymphocyte DNA synthesis inhibition, Science, 173, 1139, 1971.

2. Houck, J. C., Irausquin, H., and Leikin, S., The lymphocyte chalone, Clinical Proceedings of Children's Hospital, 28, 130, 1972.

3. Houck, J. C., Weil, R. L., and Sharma, V. K., Evidence for a fibroblast chalone, Nature New Biology, 240, 210, 1972.

4. Houck, J. C., and Cheng, R. F., Isolation, purification, and chemical characterization of the serum mitogen for diploid human fibroblasts, J. Cell. Physiology, 81, 257, 1973

5. Houck, J. C., and Hennings, H., Chalones: specific, endogenous mitotic inhibitors, FEBS Letters, 32, 1, 1973.

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
John C. Houck	20%	8,000
Richard F. Cheng	100%	16,000
Fringe Benefits @ 8.5% S & W		2,040
Technical		
None		
Sub-Total for A		26,040
B. Consumable supplies (by major categories)		
Cell cultures, media, glassware		3,600
Isotopes and chemicals		1,000
Sub-Total for B		4,600
C. Other expenses (itemize)		
Travel		500
Secretarial		250
Sub-Total for C		750
Running Total of A + B + C		31,390
D. Permanent equipment (itemize)		
Sub-Total for D		
E. Indirect costs (15% of A+B+C)		4,708
Total request		36,098

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15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	27,480	4,600	750		4,924	37,754
Year 3	29,000	4,600	750		5,152	39,502

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Protein chemistry of necrotic wounds	Office of Naval Research N00014-71-C-0203	35,000/yr	6/1/73-5/31/74
Specific mitotic inhibition of leukemic lymphocytes	National Cancer Institute CA12743	60,000/yr	1/1/72-12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Fibroblast chalone	NCI CA 14484	55,000	9/1/73-8/31/76
Immunosuppression of graft rejection by lymphocyte chalone (co-investigator)	NIH	115,000	1/1/74-12/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name John C. Houck, Ph.D.

Signature [signature] Date 7/23/73

Telephone 202 (Area Code) 835-4321 (Number) (Extension)

Checks payable to

earch Foundation of Children's Hospital

Mailing address for checks

2125 - 13th Street, N. W.

Washington, D. C. 20009

Responsible officer of institution

Typed Name Robert V. Morin

Title Executive Director

Signature Robert V. Morin Date 7/23/73

Telephone 202 (Area Code) 835-4011 (Number) (Extension)

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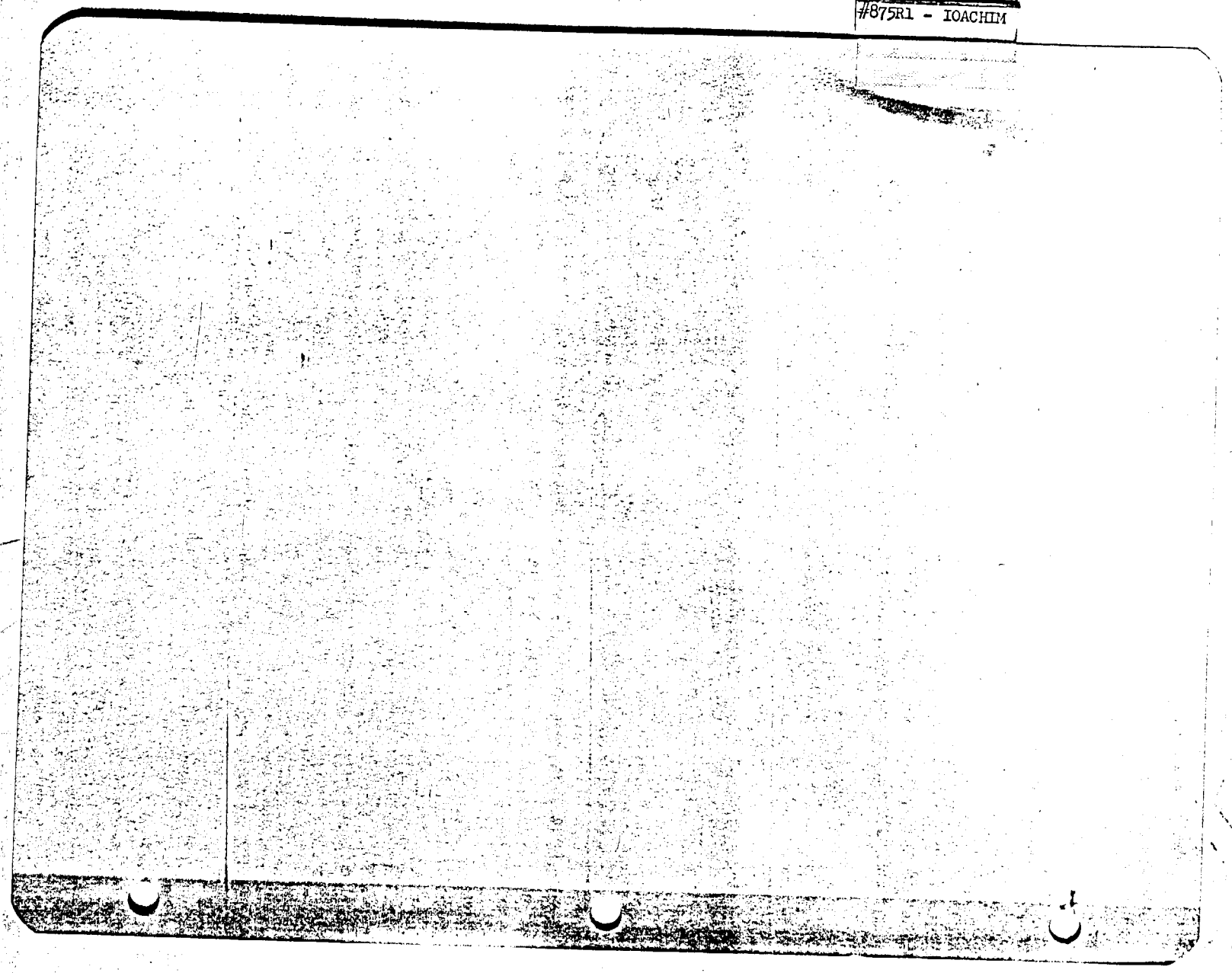

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 2, 1973

Grant Application No. 875R1
CANCER

To: The committee comprising Drs. Gardner, Huebner, and Loosli

Subject: Harry L. Ioachim, M.D., Lenox Hill Hospital, N.Y.
First renewal No. 875R1
"Studies of Human Lung and Ovarian Carcinomas"

History

Earlier grants to Dr. Sheldon C. Sommers ran from 1966 to 1968, and from 1968 to 1972.

Grant No. 875 was awarded in the amount of $90,474 (as requested); two additional years "priority in competition" were voted.

Dr. Ioachim, previously co-investigator, becomes principal investigator with the present proposal. The amount request is $79,815; no estimate for this year was provided previously.

Document Submitted

Attached is application dated July 31, 1973, incorporating summary progress report.

F.W.N.

FWN:wg
Encl.

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#775 R1

Comm.
Dr. Gardner
Dr. Huebner
Dr. Loosli

CANCER

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 31 1973

Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: July 31, 1973

1. Principal Investigator (give title and degrees):

Harry L. Ioachim, M.D.
Professor Adjunct of Pathology
College of Physicians and Surgeons
of Columbia University.

2. Institution & address:

Lenox Hill Hospital
Department of Pathology
100 East 77th Street
New York, N. Y. 10021

3. Department(s) where research will be done or collaboration provided:

Pathology

4. Short title of study:

Studies of Human Lung and Ovarian Carcinomas

5. Proposed renewal date: July 31, 1974

6. How results to date have changed earlier specific research aims:

During the first year of this grant the immunological studies of tumor-associated antigens in cancers of ovary and lung, have progressed satisfactorily resulting in several findings of interest. To follow and expand these results, major emphasis will be given in this second year to the immunological studies.

The tissue culture and electron microscopy studies, testing possible squamous metaplasia in vitro under exposure to smoke will be continued.

7. How results to date have changed earlier working hypothesis:

No significant changes in working hypotheses were made.

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8. Any additional facilities now required? Describe briefly:

Scintillation counters for beta and gamma radiations were needed, in order to explore cellular immunity in lung and ovarian carcinomas. Both scintillation counters were acquired with funds provided expressly for this research by an outside donor.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Dr. Sheldon C. Sommers has expressed the desire to act as consultant for this research project rather than principal investigator, due to limitations of time.

Dr. John A. Terzakis previously part of this project will also act as associate principal investigator.

Dr. Margaret E. Long has retired from full time activity in the Department of Pathology and will collaborate in the future as consultant with our team.

Ana Walderramus who has resigned will be replaced with Edward Paluch who has previously worked for this project on a voluntary basis.

Tove Bamberger would be a part time secretary for this grant, in charge with bookkeeping, ordering, reference filing and manuscripts writing.

10. Append outline of experimental protocol for ensuing year. - see attached protocol

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

1. - Common Antigenic Component in Ovarian Carcinoma: Demonstration by double Diffusion and Immunofluorescence Techniques - B. H. Dorsett and H. L. Ioachim - Immunological Communications, 2: 173-184, 1973
2. Antigenic and Morphologic Properties of Ovarian Carcinoma -H. L. Ioachim. B. H. Dorsett, M. Sabbath, B. Andersson and H. R. K. Barber- Gynecologic Oncology, 1:000, 1973.
3. Antigenic Component Associated with Human Ovarian Carcinoma- B. H. Dorsett, M. Sabbath and H. L. Ioachim- Feder. Proc. pp. 4442, 1973.
4. Neurosecretory Appearing Cells of Human Segmental Bronchi-J. A. Terzakis Sheldon C. Sommers and B Andersson - Lab. Investigation 26:127, 1972
5. Ultrastructure of human endometrial adenocarcinoma-J. A. Terzakis, S. C. Sommers and B. Andersson-(in progress).
6. Age Changes in Ultrastructure and Histochemistry of Human Bronchi- M. A. Long, B. Andersson, J. A. Terzakis and S. C. Sommers (in preparation)

12. Summary progress report (append in standard form as separate document, unless recently submitted).

see attached report

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13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Sheldon C. Sommers M.D. - Consultant	10%	none
Harry L. Ioachim M.D. - Principal Investigator	25%	none
John A. Terzakis M.D. - Associated Principal Investigator	25%	9,000
Margaret E. Long Ph.D. - Consultant	10%	2,500
Elena C. Schmidt Ph.D. - Investigator	50%	10,000
Brent H. Dorsett B.S. - Investigator	100%	11,500
Technical		
Marjorie Rosenberg B.S.	100%	10,000
Antonia Pearse B.S.	30%	3,000
Edward Paluch	30%	3,000
Tove Bamberger - Secretary	30%	3,000
	Sub-Total for A	52,000
B. Consumable supplies (by major categories)		
Reagents (immunology)		1,000
Tissue culture media		1,000
Glassware, isotopes, photomaterial		1,500
Animals and animal food		1,000
Electron Microscopy (supplies and services)		6,000
	Sub-Total for B	10,500
C. Other expenses (itemize)		
Travel		1,600
Publication costs, illustrations, mailing, miscellaneous		2,000
	Sub-Total for C	3,600
	Running Total of A + B + C	66,100
D. Permanent equipment (itemize)		
1. Swinging Bucket Rotor Type SW 41 Ti for ultracentrifuge		2,750
2. Electrophoresis apparatus		650
3. Immunoelectrophoresis unit		400
	Sub-Total for D	3,800
E. Indirect costs (15% of A+B+C)	E	9,915
	Total request	79,815

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Harry L. Ioachim, M.D.

Signature [signature] Date July 31, 1973

Telephone 212 (Area Code) 794-4420 (Number) (Extension)

Checks payable to

Lenox Hill Hospital

Mailing address for check:

100 East 77 Street
New York, N.Y. 10021

Responsible officer of institution

Typed Name Louis F. Parker

Title Vice President

Signature [signature] Date 7/31/73

Telephone 212 (Area Code) 794 (Number) 5141 (Extension)

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ADDENDUM

10. Outline of Experimental Protocol for Ensuing Year

A. Tumor-associated antigens of lung tumors.

Bronchial squamous cell carcinoma representing over 50% of all lung cancers is the histologic type generally believed to be associated with smoking and air pollution. Squamous cells, normal and neoplastic, contain numerous doubly refractive tonofibrils forming a tridimensional framework around the nucleus. The tonofibrils contain sulfhydril and disulfide groups and are assumed to be the precursors of keratin. When keratin, a poorly soluble substance, comes into direct contact with mezenchymal tissues, a granulomatous inflammation is elicited, characterized by the accumulation and proliferation of reticulo-endothelial cells. A crucial point in these types of reactions is to differentiate between granulomatous hypersensitivity and response to a colloidal foreign body substance. In our preliminary studies the local reaction to keratin was accompanied by a humoral response in which sera of animals injected with keratin reacted specifically with keratinized squamous epithelial cells. As part of our study of tumor-associated antigens it was decided to analyze this reaction to keratin in order to apply it subsequently to the detection of keratin-forming squamous cell carcinomas.

At a later stage radioactive labeling of gammaglobulins will be attempted and their attachment to squamous cells of the epidermis as well as to squamous cell carcinoma followed by immunofluorescence and electron microscopy. At the same time, sera of patients with squamous cell carcinoma will be tested for reactivity against squamous cells and extracts thereof.

B. Tumor-associated antigens of ovarian carcinoma.

Purification of the antigenic component found in ovarian carcinoma will be undertaken with the use of gel filtration and ion exchange chromatography, alongwith preparative ultracentrifugation. The highly purified antigen thus obtained will be used to produce monospecific antisera by heterologous immunization. It is to be assumed that ovarian carcinoma antigens in the circulating blood of patients will be in almost infinitesimal amounts, therefore making the availability of very sensitive

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techniques a prerequisite for their detection. The production of highly purified antigen preparations and antisera will be necessary for the development of such sensitive techniques. Radioimmunoassay procedures as the most sensitive techniques presently in use, will be developed for the search of ovarian carcinoma antigens in the peripheral blood of tumor patients. In addition , the purified antigenic preparations will be used to test the cellular immune system of these patients. For this purpose we are presently adapting the macrophage migration inhibition test and the lymphoblast transformation assay using our new liquid scintillation counter.

C. Squamous metaplasia induced by smoke in cultures of epithelial tumors.

The cultures subjected to the effects of smoke during the first part of this study will now be processed for light and electron microscopy and studied for possible cytologic changes. Keratin-forming squamous epithelium will be grafted subcutaneously in rats and guinea-pigs and the grafts will be removed at various intervals from 1 hour to 30 days. The tissue reaction to the graft will be studied in light and electron microscopy.

Extracts of keratinized squamous epithelium will be prepared and used to immunize rabbits according to methods previously described. Sera reacting with extracts of squamous epithelium in gel immuno-diffusion assays will be fractionated for isolation of gammaglobulins.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

ADDENDUM

12. Summary Progress Report.

A. Tumor-associated antigens of lung tumors.

The investigation of antigens associated with carcinomas of the lung began during the first year of this grant in accordance with a protocol similar to that used in the previous work on ovarian carcinoma. Heterologous sera obtained from rabbits immunized with lung tumor extracts were absorbed and tested in gel immunodiffusion and immunofluorescence assays. The results were not as specific and consistent as in the tests of ovarian carcinomas. New animals have been therefore immunized with better purified extracts and according to different schedules of immunization.

In addition, a new line of investigation has been developed. It is a common observation that squamous cell carcinomas freqently elicit granulomatous reactions due to their content of keratin. In the lung as well as in other locations, keratin, a poorly soluble substance produced by squamous cell carcinomas, induces an inflammatory process mediated through the accumulation of reticulo-endothelial cells, similar in many respects to a hypersensitivity reaction. The possibility that this reaction might be accompanied by general humoral or cellular events, which then could be used for its detection, stimulated our interest for its study.

The granulomatous inflammatory reaction was reproduced experimentally by the introduction of portions of keratinized epidermis in the subcutaneous tissue of rats and guinea pigs. The local tissue reaction was studied histologically at various intervals and the sera of these animals assayed against squamous epithelia in immunofluorescence tests. Positive specific immunofluorescent reactions were consistently obtained with sera of sensitized animals against keratin and keratohyalin-containing squamous cells, including components of squamous carcinomas.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrm0037

B. Tumor-associated antigens of ovarian carcinomas.

The need for immunological diagnosis in carcinoma of the ovary, a tumor that is inoperable in 60% of the patients at the time of clinical diagnosis, has been stressed repeatedly. During the first year of this grant, earlier studies on the culture in vitro and immunology of ovarian carcinoma have been continued and expanded. The ultrastructure of various types of ovarian tumors has been examined and a total of over 70 carcinomas of the ovary grown in tissue cultures for intervals of up to 10 months. A bank of lyophilized extracts of different types of ovarian tumors has been established in our laboratory. Heterologous antisera to pools of ovarian tumor extracts were raised in rabbits and then extensively absorbed with normal human tissues including normal ovaries, and serum components. These highly absorbed antisera assayed in gel immunodiffusion tests against a large variety of normal and neoplastic tissue extracts detect specifically and consistently the ovarian carcinoma extracts.

In indirect immunofluorescence assays both ovarian carcinoma tissue cultures and ovarian carcinoma cell suspensions, show specific cytoplasmic staining with the absorbed heterologous antisera in contrast to normal ovaries, benign ovarian tumors and unrelated malignant human tumors.

These results indicate the existence of a common, probably cross reacting, antigenic component shared by different ovarian carcinomas. The concentration, purification and characterization of this antigenic component is attempted at the present time.

The granulomatous inflammatory reaction was reproduced experimentally by the intradermal portions of formalinized epithelial

C. Squamous metaplasia induced by smoke in cultures of epithelial tissues

According to our proposal, cultures of various epithelial tissues, were submitted to periodical smoking using a standard CTR type smoking apparatus. The cultures used, included normal embryonal human lung and kidney, normal embryonal rat lung and kidney, normal human amnion, ovarian carcinoma, mammary carcinoma and lung adenocarcinoma. After the lethal toxic dose was established, smoking of these cultures was performed twice a week for up to four months. Coverslips of smoked cultures and matched controls were removed at similar periodical intervals, fixed, stained and examined under the light microscope. Similarly, smoked and control cultures were fixed and embedded for electron microscopy.

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#603C - KAUFFMAN

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 12, 1973

Grant Application No. 603C

To: The committee comprising Drs. Andervont, Gardner, Loosli, and Sommers
Shirley L. Kauffman, M.D., State University of New York, Downstate
"Pulmonary Alveolar Hyperplasia Induced by Carcinogens"

History

This investigator has been supported by CTR since 1965.

Last year Dr. Kauffman requested a three year grant at some $37,000 a year. She was awarded one year without further commitment, at $25,000 per annum. Therefore, the enclosed application competes as a new request.

Application No. 603C requests $30,521 for 1974, plus one additional year. Also requested is approximately $7,500 interim support for October 1, 1973 through December 31, 1973.

Documents Submitted

Attached are letter dated July 9, 1973 and application dated July 6, 1973.

FWN

F.W.N.

FWN:wg
Encls.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

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gk

STATE UNIVERSITY
OF NEW YORK
DOWNSTATE MEDICAL CENTER

• DEPARTMENT OF PATHOLOGY

July 9, 1973

JUL 11 1973

Dr. Robert C. Hockett
The Council for Tobacco Research U.S.A.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

I have forwarded a grant request for $30,521 through the Research Foundation, Albany, and expect that it should be returned to you by July 15th. A copy of this request is enclosed. Since my current grant expires on September 31, 1973 and new grants are activated as of January 1, 1973, I would like to request that, if my proposal is funded, I receive an additional adjustment for the 3 months remaining in the year, or approximately $7500 including overhead.

Thank you for your consideration of my request.

Sincerely,

Shirley L. Kauffman, M.D.
Professor of Pathology
Department of Pathology

MAILING ADDRESS: 450 CLARKSON AVENUE, BROOKLYN, NEW YORK 11203 / PHONE: (212) 270-1000

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Comm.
Dr. Andervont
Dr. Gardner
Dr. Loosli
Dr. Sommers

CARCINOGENESIS

No. 603C

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: July 6,1973

RECEIVED
JUL 11 1973

1. Principal Investigator (give title and degrees):

Shirley L. Kauffman, M.D.
Professor of Pathology

2. Institution & address:

State University of New York
Downstate Medical Center
450 Clarkson Avenue,
Brooklyn, N.Y. 11203

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Pulmonary Alveolar Hyperplasia Induced by Carcinogens

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 2 years

7. Brief description of specific research aims: The general aim of the present study is to adapt morphometric methods, developed for analysis of growth of neonatal lung populations, to analysis of the preneoplastic growth phase induced by chemical carcinogens. The specific questions to be answered are:

1. Does alveolar epithelial hyperplasia occur following exposure to lung carcinogens other than urethane and if so, is the time sequence of the hyperplasia fixed?

2. Is there a relationship between aging and Type II cell hyperplasia which might underlie the finding that spontaneous tumors commonly occur in old mice.

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Source: https://www.industrydocuments.ucsf.edu/docs/ntcm0000

8. Brief statement of working hypothesis: 2.

The hyperplastic growth phase of Type II alveolar cells demonstrated in mice continuously exposed to urethane in drinking water (1) may be a general phenomenon reflecting, in part the augmentation of "preneoplastic" cells. As such, it should be demonstrable following exposure of mice to injections of a variety of lung carcinogens. This may be a suitable model for study of early changes in carcinogens, as well as a possible biologic essay method for pulmonary carcinogen testing.

9. Details of experimental design and procedures (append extra pages as necessary)

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Details of Experimental Design and Procedure

1. Relation of Hyperplasia to Neoplasia

Chimken and Pollisar (2) described hyperplasia of "lung cells" following a single injection of urethane. This preceded the development of lung tumors, and disappeared as tumors developed. Refinements of sectioning technique and use of morphometric autoradiographic method (Progress report Spring '73) have made it possible to identify the cells involved in the hyperplasia. These are the Type I and II alveolar cells, the latter the cell of origin of lung adenomas. The time sequence of this hyperplasia in mice continuously exposed to urethane in drinking water is shown in Figure 1 and 2. It has been suggested that hyperplasia may be dependent on the continuous presence of carcinogen (discussion macrophage Conference, The Council for Tobacco Research, N.Y. December 1972) and hyperplasia itself may amplify tumor yield by virtue of its associations which increased cell division. In order to control this factor, single injections of carcinogens will be used in the following experiments.

Experimental Plan

Adult mice will be exposed by single injection to 3 different pulmonary carcinogens, urethane, DMBA (9,10-Dimethyl-1,2 benzanthracene) and MNU (methyl nitrosourea) and the reaction of their pulmonary epithelium at intervals after injection will be analyzed by quantitative methods. Additional groups of injected and control animals will be killed at

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Details of Experimental Design and Procedure

10 months and the number of lung tumors counted. Table I summarizes the carcinogen, dose, weeks for sacrifice and incidence of tumors reported at 10 months along with references from which the data on dose and tumor yield were obtained.

TABLE FOR CARCINOGEN EXPOSURE IN ADULT MICE

Carcinogen	Dose/Body wt.	Sacrifice for Morphometry (Weeks)	Sacrifice for Tumors % Tumors	Reference
Urethane	1.0 mg/g	1,2,3,4,5,6	90% at 10 m	This laboratory
MNU	50.0 µg/g	1,2,3,4,5,6	100% at 10 m	(3)
DMBA	10.0 µg/g	1,2,3,4,5,6	100% at 10 m	(4)
Control	Saline	1,2,3,4,5,6	< 5% at 10 m	This laboratory

Method of Tissue Preparation

Mice will be anesthetized at stated intervals, a tracheostomy tube inserted for glutaraldehyde instillation and a bilateral pneumothorax performed immediately before instillation. After 2 hour fixation, the mediastinal contents will be dissected from the lung and lung volume measured by water displacement. Multiple 1 mm cubes of lung tissue will be processed for Epon embedding and subsequently cut at 1.5 micra, placed on glass slides and stained with Toluidene Blue.

Method of Tissue Analysis

Slides will be coded and examined in the Wild M-501 sampling microscope; identity and the numbers of cells in each microscopic field will be determined in 60-100 test areas from each animal's

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Details of Experimental Design and Procedure

lung (3 animals/group). The number of Type I and II cells/cm^3 of lung can be calculated for each animal using the relation $Nv=Na/_D$ where Nv is the number of nuclei per unit volume, Na is the number of nuclei per unit section area, and D is the nuclear diameter. The number of Type I and II nuclei can then be calculated by multiplying the numerical density by the volume of lung parenchyma. (5)

The final groups (12 animals/groups) will be killed after 10 months and the number of superficial adenomas in the whole lung, and in serial section of one lobe will be determined.

These experiments should answer the following questions:

1. Is the pattern of alveolar epithelial hyperplasia which is found with continuous urethane exposure also found after a single urethane injection?

2. Do the carcinogens MNU and DMBA elicit a hyperplasic response?

3. If so, does the time sequence vary with the carcinogen and can it in any way be related to the final number of tumors produced?

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

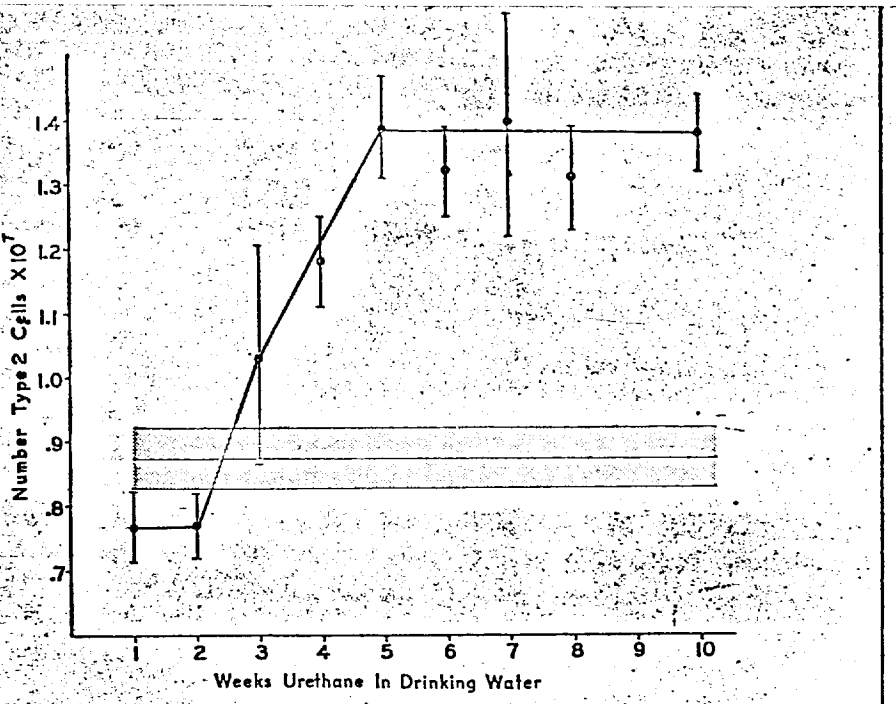

Figure 1

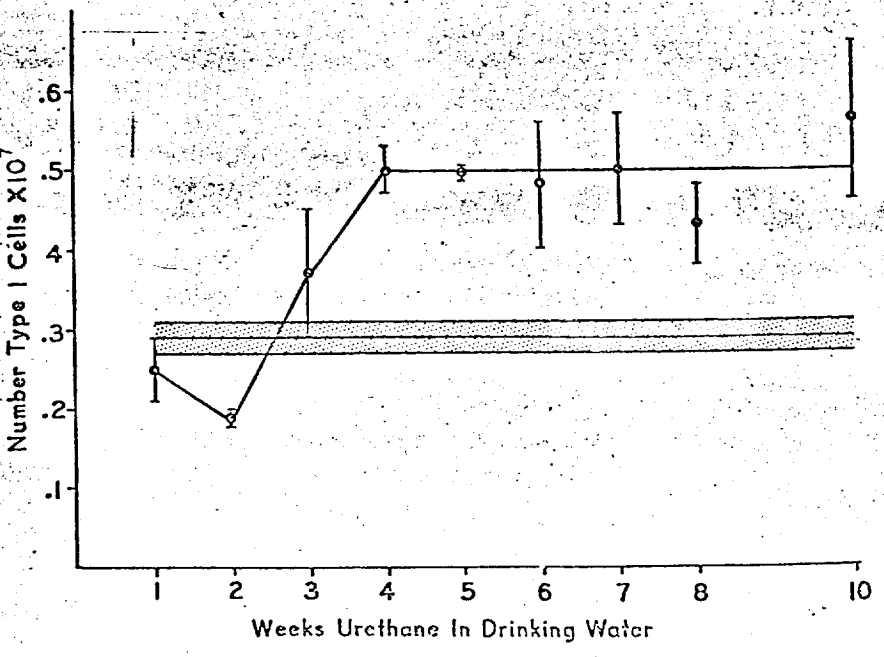

Figure 2

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Details of Experimental Design and Procedure

2. Life Cycle of epithelium in relation to Hyperplasia

Knowledge of Type II cell cycle and the growing evidence that Type II cells are probably the stem cell of alveolar epithelium (6, 7, 8) introduces the question of maturation and differentiation of this system. If the Type I cell arises from Type II stem cell, and a steady state is maintained, then some mode of exit of Type I cells from the population would be expected.

Alternately, Type I cells may not constitute a steady-state system and may have no turnover. In the latter case Type I cell population would increase throughout the life of the individual. No previous study has investigated alveolar epithelium as an expanding population. The question as to whether increase in cell numbers or hyperplasia may be a natural component of aging could be answered by a study of population kinetics.

Two types of experiments will be done:

A) Aging Experiment

In this long term experiment, lungs of mice from 6 months to 24 months of age will be studied using autoradiographic and morphometric methods to quantitate cell population numbers and obtain labeling indices.

Swiss Webster mice will be maintained under normal conditions for a maximum period of 16 months, with groups sacrificed at 4 month intervals, beginning at 6 m of age. ^{3}H thymidine (1 μc/gram:3.0 c/mM) will be injected into mice 60 minutes prior to sacrifice and tissue will be fixed and processed for autoradiography and morphometry

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Details of Experimental Design and Procedure

according to methods previously described.

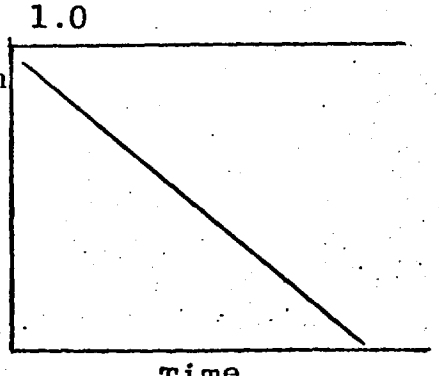
The labeling index of Type II cells and the total number of Type I and II cells in lung will be determined in order to detect any increase in number of cells with age, or a decrease in proliferation of the Type II stem cell with age.

B) Turnover of Type I cells

In this experiment, an attempt will be made to label the precursor cells of Type I epithelium, and follow the persistence of Type I cells by content of radioactivity. Since Type I cells do not normally divide they should maintain their radioactivity throughout their life time. The mean grain count and labeling index of Type I cells will be used to measure their survival.

Labeling of Precursor cells

Epithelium of 17 day mouse lung has a cycle time of approximately 10 hours (8) so that it should be possible to label all epithelium by spaced injections of ^{3}H thymidine over a 24 hour period. The first generations of Type I cells, produced from labeled Type II stem cells will be labeled; and the fraction labeled will decrease at a rate equal to the production of Type I from unlabeled precursors.



The slope of the fraction of labeled Type I cells will thus represent the rate at which new (unlabeled) Type I cells are produced.

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Details of Experimental Design and Procedure

The life span can be estimated by the mean grain count of Type I cells. Since Type I cells are not known to divide under normal conditions, any Type I cell produced from labeled Type II cell should maintain its mean grain count over its lifetime. The mean grain count of Type I cells should not change unless there is turnover of Type I cells. These 2 experiments should answer the questions as to whether alveolar epithelium increases with age and whether Type I epithelium is a renewal or an expanding population.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

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2. Shimkin, M.B. and Pollisar, M.J. Some quantitative observations on the induction and growth of primary pulmonary tumors in Strain A mice receiving urethane. J. Natl. Cancer Inst. 16: 75-97 1955

3. Frei, Jaroslav V. Toxicity, Tissue Changes, and Tumor Induction in Inbred Swiss Mice by Methyl nitrosamine and -amide compounds. Cancer Res. 30:11-17 1970

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5. Kauffman, S.L. , Burri, P.H. and Weibel, E.R. The Post Natal Growth of the Rat Lung. To be submitted. Anat. Rec. 1973

6. Kapanci, Y., Weibel, E.R., Kaplan, H.P. Pathogenesis and reversibility of the Pulmonary lesions of oxygen toxicity in Monkeys. II Ultrastructural and Morphometric studies. Lab. Invest. 20: 101 1969

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8. Evans, M.J., Cabral, L.J., Stevens, R.J. and Freeman, G. Renewal of Alveolar Epithelium in the Rat Following Exposure to NO_2. Amer. J. Path. 70: 175 1973

9. Kauffman, S.L. Cell cycle times in developing mouse lung - in preparation.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

General laboratory, animal rooms, electron microscope laboratory, dark rooms for autoradiography, Wild sampling microscope and morphometry equipment.

11. Additional facilities required: None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

13. Publications

1. Kauffman, S.L., Burri, P.H. and Weibel, E.R. Post Natal Growth of the Rat Lung. II Morphometric-Autoradiographic Analysis to be submitted Anat. Rec. 1973

2. Kauffman, S.L. Kinetics of Alveolar Epithelial Hyperplasia in Lungs of Mice Exposed to Urethane. 1. Morphometric Analysis of Type I and 2 Cell populations. submitted for publication. Lab. Invest. 4/73.

3. Kauffman, S.L. Alterations in Cell Proliferation in Mouse Lung Following Urethane Exposure III Effects of Chronic Exposure on Type 2 Alveolar Epithelial Cell. Amer. J. Path. 68:317-325 1972

4. Kauffman, S.L. Alterations in Cell Proliferation in Mouse Lung Following Urethane Exposure. II. Effects of Chronic Exposure on Terminal Bronchiolar Epithelium. Amer. J. Path. 64:531-539 1971

5. Kauffman, S.L. Alterations in Cell Proliferation in Mouse Lung Following Urethane Exposure I The Non-Vacuolated Alveolar Cell. American J. Path. 54: 83-93 1969

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

R: REDACTED MATERIAL

BIOGRAPHICAL SKETCH

NAME: Shirley L. Kauffman, M.D.

DATE OF BIRTH: REDACTED

PLACE: REDACTED

EDUCATION:
Northwestern University 1942-44
University of Chicago, B.S. 1946
University of Chicago, M.S. 1948
University of Kansas, M.D. 1955

Hospital Appointments
Provisional Assistant Pathologist, The New York Hospital, July 1, 1955 - June 30, 1957
Trainee of National Cancer Institute assigned to Francis Delafield Hospital, July 1, 1957 - June 30, 1959.
Pathologist, Kings County Hospital 1961 - present
Pathologist, State University Hospital 1966-present
Consultant VA Hospital, Brooklyn 1973

Research and University Appointments
Bacteriologist, U.S. Govt. Food and Drug Administration, 1948 - 1949.
Bacteriologist, University of Chicago, 1950-1951
Assistant in Pathology, Cornell University Medical College, July 1, 1955-June 30,1957
Instructor in Pathology, Albert Einstein Medical College, July 1, 1959-1960
Fellow in Pathology, July, 1960 - December 1960 State University of New York
Assistant Professor, State University of New York January 1, 1961 - 1966
Associate Professor, State University of New York, April 1966 - 1970
Visiting Professor, Dept. of Anatomy, University of Bern, Switzerland, July 1969 - December 1969
Professor of Pathology, State University of New York, September 1970 -

License
American Board of Pathology 1960
License, New York State ; 1957

Society Membership

REDACTED

REDACTED

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)

		% time	Amount
Technicians	I. 10,000		
	2. 8,000		
	18,000		
	.(18)		18,000
			+ 3,240
			21,240

Technical

Sub-Total for A _____

B. Consumable supplies (by major categories)

Fixatives, materials for Epon embedding, slides	800
Glass & Diamond knives and sharpening	1000
Liquid emulsion, isotopes carcinogens	2000
Animals & maintenance	800
	4500

Sub-Total for B 4500

C. Other expenses (itemize)

Photographic film for morphometric analysis	300
Travel to meetings	300
Publication Cost (medical illustration, reprints)	500

Sub-Total for C 800

Running Total of A + B + C 26,540

D. Permanent equipment (itemize)

None

B.K.
R.C.H.

Sub-Total for D 26,540

E. Indirect costs (15% of A+B+C) Overhead 15% of ABC + 3,981

Total request $30,521

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	22,515	4800	800		4217	32,330
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Morphometric Study of Mouse Lung Exposed to Carcinogens	The Council for Tobacco Research. U.S.A. Inc.	25,000	Oct.1-Sept.1973 1972

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Pulmonary Carcinogenesis in Fetal and Neonatal Lung	National Institute of Health	$41,000	Oct. 1,1973- Sept. 31,1976

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Acting Chairman

Checks payable to

Mailing address for checks

Principal investigator

Typed Name Shirley L. Kauffman

Signature Shirley L Kauffman Date 7/7/73

Telephone 212 270-1305
Area Code Number Extension

Responsible officer of institution

Typed Name

Title

Signature Date

Telephone
Area Code Number Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

#572C - LEVY

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 25, 1973

Grant Application No. 572C

To: The committee comprising Drs. Andervont, Gardner and Huebner
Jay A. Levy, M.D., University of California, San Francisco
Continuation application No. 572C
"Development of a Model System In Vitro for Studying Carcinomas"

History

CTR Grant No. 572, with Dr. T. Timothy Crocker as principal investigator, began early in 1967. Dr. Jay A. Levy joined the program several years later to strengthen the virology.

As a result of Dr. Crocker's move to Irvine Dr. Levy became principal investigator as of October 1972 in order to finish the work then pending.

Application No. 572C is entirely Dr. Levy's proposal. It requests $49,119 plus one additional year. The same technical staff as heretofore are budgeted.

Document Submitted

Attached is application dated 7-17-73.

Comment

Dr. Hockett will comment on program priority of this proposal. Dr. Kreisher will provide a scientific evaluation.

F.W.N.

FWN:wg
Encl.
cc: Drs. Hockett and Kreisher

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Source: https://www.industrydocuments.ucsf.edu/docs/ncm0000

#572c

Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED JUL 2 3 1973

Application for Research Grant
(Use extra pages as needed)

Date 7-17-73

1. Principal Investigator (give title and degrees):
Jay A. Levy, M.D., Assistant Clinical Professor of Medicine, Dept. of Medicine and Research Associate, Cancer Research Institute.

2. Institution & address:
University of California, 3rd & Parnassus Avenues, San Francisco, Calif. 94143

3. Department(s) where research will be done or collaboration provided:
Cancer Research Institute

4. Short title of study:
Development of a model system in vitro for studying carcinomas.

5. ~~Proposed starting date:~~ September 1, 1973

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

We are endeavoring to obtain a line of mouse epithelial cells which is susceptible to transformation by viruses and chemicals. We shall study the steps involved in epithelial cell transformation and the combined effects of chemicals and viruses on this transformation.

The experience and knowledge gained in the cultivation and study of mouse epithelial cells will be used for research on human epithelial cell transformation.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

8. Brief statement of working hypothesis:

Over 80% of human cancers, particularly those of the lung, are carcinomas -- arising from normal epithelial cells. Nevertheless, most of the in vitro systems presently employed to study carcinogenesis use mesenchymal or fibroblast cells and transformation is defined by the production of sarcomas. We have been attempting over the past 2 years to develop a model system in tissue culture for studying epithelial cell transformation. It seems reasonable that epithelial cells will better reflect the malignant changes occurring in vivo, resulting in the production of carcinomas. Moreover, once an epithelial line is established, various contaminants in our atmosphere can be checked for their carcinogenic potential.

9. Details of experimental design and procedures (append extra pages as necessary)

Introduction and Background

It has become evident from data obtained in several laboratories that certain chemicals are capable of inducing carcinomas in vivo (1-3). This induction may lead to the production of specific RNA tumor virus antigens in the tumor (2) and sometimes to a "C" type virus as well (3,4). These observations suggest that chemicals and virus may play a concerted role in the ultimate transformation of some cells.

Similar studies in tissue culture have shown that pretreatment of rodent cells with RNA tumor viruses may increase or help initiate the transforming ability of certain chemicals (5-12). In certain situations the chemical may activate endogenous C-type virus in the tissue culture cells (13,14). In other reports, chemicals transform cells but the possible role of an RNA tumor virus in this transformation has not been examined (15-21). In most cases, however, the cells transformed have been fibroblasts which form sarcomas after inoculation into susceptible hosts. These type tumors do not truly reflect the in vivo experiments leading to carcinoma production. Transformation of rat epithelial cells in tissue culture with subsequent production of carcinomas has been reported (22,23). The possible production of carcinomas by mouse cells transformed in vitro has been suggested (24,25) but not confirmed. One of the main deterents to achieving a reproducible in vitro system for testing carcinoma induction has been the difficulty in obtaining epithelial cell cultures which maintain their differentiation.

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Our research is aimed at developing such an in vitro technique. We have chosen the mouse system not only because of the readily available inbred strains but because it is a well defined mammalian cell system and has indigenous and exogenous RNA tumor viruses which have been more carefully and more extensively studied than C-type viruses associated with other animals. Moreover, mouse cells appear to be more readily transformed by chemical than other rodent cells (11). Using mouse cells, then, we can study the events leading up to chemical carcinogenesis as well as the interrelationship of C-type viruses with this transformation event. Our chief objective at first is the establishment of a pure epithelial cell line. Details of our previous attempts were presented in our Annual Report to the Council in 1972. We have learned by these early efforts that the cell morphology in tissue culture is not an accurate method of characterizing cell type. These conclusions are illustrated as well in the results of Sanford, et al. who noted the transformation of epithelial cells to fibroblast-like cells and vice versa in tissue culture (26-28). The best technique

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is to transform the cell first and then note the kind of tumor (epithelial or mesenchymal) it produces on inoculation into specific animal hosts.

Our basic procedure has been to obtain epithelial cells from mouse tracheas or skin by standard techniques (29, see Annual Report, 1972) and cultivate those cells which have the morphology of epithelial cells (see Fig. 1-3). Recently we have used a method of floating the skin of newborn mice on top of a trypsin layer (0.25%) overnight. The next day the epithelium can be separated with forceps, and the residual dermis can be removed easily with scalpel and forceps. This technique has given us excellent epithelial cell islands both from mouse skin and mouse tracheas.

In attempts to cultivate these epithelial cells, many different media have been tried including McCoys 5a, RPMI 1640, RPMI 1629, Medium 199 as well as the addition of other growth factors such as vitamin A, sodium pyruvate, and non-essential amino acids. We feel many other potential growth factors should be tried. Attention must be given as well to the percentage of serum in the medium. We have seen that epithelial-like cells, grown in medium containing 10% or 20% fetal calf serum, survive longer than those grown in 2%, but their morphology changes to a more fibroblastic type.

Similar to our experience with NIH Swiss cells, BALB/c mice epithelial-like cells have been easily established in culture when serum levels of 10-20% were employed. These cells show characteristics of contact-inhibition but on transformation they produce only sarcomas in recipient hosts. Similar results have been reported from other laboratories trying to obtain epithelial cell lines (30). We believe strongly, that epithelial cells when encouraged to grow rapidly will lose their differentiation and become fibroblastic.

For this reason we propose to continue those efforts in which factors are added to the media that may maintain cell differentiation and give some stimulation of growth. The tissue culture requirements of primary epithelial cells are obviously different from those defined for standard tissue culture cells and must be specifically determined.

Recently we have isolated a clone of NZB cells from NZB embryos which morphologically resemble epithelial cells (Figs. 4-5). These NZB cells are very sensitive to chemicals and can be transformed with as low as 0.05 µg of 3-methyl cholanthrene (3-MC). A typical focus of cell alteration is illustrated in Fig. 6. The NZB cell line is known to contain a C-type virus but very little infectious virus is produced. Pretreatment with benzanthracene (BA), a relatively non-carcinogenic hydrocarbon, appears to increase the sensitivity of this cell line to chemicals so that detection of small amounts of carcinogen in the atmosphere may be achieved. Although this cell line also produces sarcomas, not carcinomas, in mice (Arnstein, unpublished data), we feel this line holds promise for helping us understand the steps leading up to a transformation event by chemicals. In this sense, the cell line will continue to be useful while we try to establish primarily epithelial cells in culture for subsequent chemical transformation studies.

Methods of Procedure

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A. Establishment of epithelial cell lines

1. Tracheas from weanling mice or skin from newborn BALB/c mice will be used since cell inoculation and transplantation experiments are best achieved using an inbred strain. We had originally found that only two 60 mm petri dishes containing epithelial cell islands can be obtained after the trypsinization of 10 mouse tracheas using the technique of differential trypsinization. This method involves exposing the primary plates to 0.25% trypsin for a long enough time period to remove

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preferentially scattered fibroblasts without removing the majority of small clusters of epithelial cells which are less sensitive to trypsin and remain attached to the dishes. Islands of epithelial cells which remain are selected for further propagation (Fig. 1-3).

By using a new technique of floating the tracheas or skin on top of a trypsin layer we have been able to double or triple this recovery of epithelial-like cells. The method includes an overnight incubation at 4°C and then removal of the dermis, which separates easily with forceps and scalpel. Many islands of epithelial-like cells can be observed 24 hours after plating. Difficulties, however, arise when we try to establish these cells as continuous lines. Certain growth factors, such as the following will be added to the different media employed.

(a) Vitamin A: This vitamin has been demonstrated as an important factor in maintaining epithelial cell differentiation (31). It encourages epithelial growth in wound healing (32).

(b) Cortisone: Studies by Tomkins and his coworkers indicate that steroids such as dexamethosone are strong inducers of protein production (33). It is also a known inhibitor of fibroblastic growth in vivo (34).

(c) Estrogen

(d) Cyclic AMP

(e) Progesterone: Reboud and Pageaut (35) found that progesterone given to C57 Black mice led to an increase in 20-MC induced cervical carcinomas. The effect of this hormone may be related to a stimulation of cervical epithelial cell growth. It will be interesting to see whether its presence in culture medium will influence epithelial cell growth. The compound may also be effective as a co-carcinogen in vitro as it appears to be in vivo.

(f) Isoproterenol: This compound stimulates DNA synthesis (36).

2. Besides the above method for the cultivation of epithelial cells, a new procedure would be attempted as described below. This method calls for a selected media (medium D) which can be prepared by Grand Island Biologicals (37).

Sequential passage method: Adult tracheas, liver, or skin from mice will be minced and trypsinized (0.25%) for 10 min in a glass flask using a magnetic stirring bar. The tissue fragments will be allowed to settle down and then the cells floating in the supernatant will be collected. A repeat trypsinization of the tissue fragments will be done for an additional 15 minutes and the fragments allowed to settle. The supernatant will then be added to the first collection, and this mixture poured over a nylon gauge (to remove large tissue fragments) into centrifuge tubes. The cells will be centrifuged and gently washed twice; they will then be plated in 75 cm^2 plastic tissue culture flasks. These flasks will be placed in an incubator at 37°C for 20 minutes. After this time period, the medium containing unattached cells will be transferred from each flask to a second flask. The first flask will be refed with medium. The medium from the second flask is then removed after 20 min. and placed in a third flask. This last flask is allowed to remain in the incubator for 2 hours before the medium is withdrawn and discarded. All the flasks are refed with Medium D containing 10% FCS. After 24 hours any fibroblast cells found in the third set will be destroyed by a flamed platinum wire. The first and second flasks are examined for evidence morphologically of epithelial cell growth. Those flasks with predominantly epithelial cells will be saved.

By this method, the fibroblasts are found to attach earliest and by the third passage the majority of the cells should be epithelial-like. Any contaminating fibroblast cells can be removed by the flamed platinum wire. This technique not

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only permits recovery of primarily epithelial cells, but also avoids the selection of certain epithelial cells. Such a selection probably results from our other procedures in which small islands of epithelial-like cells are isolated for further propagation.

Results with rat liver cells has shown that an original inoculation of 10^8 cells/flask results in good epithelial cell recovery by the third flask (37). We shall have to experiment with different cell concentrations to determine the optimum for mouse cells.

B. Determination of Cell Type in Tissue Culture.

1. Our most sensitive method for defining a cell type in tissue culture is to transform the tissue culture cells and observe the type of tumor they produce in recipient animals. It is conceivable, however, that epithelial cells lose their differentiation when inoculated into mesenchymal areas of the animal host, and therefore only sarcomas are produced. The same cells might maintain their differentiation if inoculated into animals in areas where epithelial growth normally takes place. For this reason, we are collaborating with Dr. Stuart Yuspa at NIH (see attached letter) and Dr. Arthur Furst in San Francisco. Dr. Yuspa will inoculate our transformed "epithelial-like" cells onto the back of irradiated recipient mice, and note the type of tumors which develop. In preliminary studies with his technique, Dr. Yuspa has been able to transplant skin successfully from various mouse strains (39).

2. Dr. Arthur Furst and his coworkers have demonstrated the production of tumors in the alveoli of mouse lungs after instillation of cell suspensions into the lungs. The alveolar area is an excellent epithelial area for the cultivation of tumors. It is conceivable that transformed epithelial cells will grow into carcinomas more readily in this environment.

3. Histochemical tests to determine the presence or absence of certain cellular enzymes will be conducted. Dr. Clayton Loosli has agreed to help in these studies. Samples of each cell strain will be plated on coverslips at low concentrations to provide colonies of epithelial-like or fibroblast cells. In Dr. Loosli's laboratory in Los Angeles, these colonies will be tested by selected histochemical stains for alkaline phosphatase, glucose-6-phosphate dehydrogenase (G6PD), and lactic dehydrogenase (LDH). With frozen sections of hamster and mouse lung, Dr. Loosli has demonstrated the following differences: Alkaline phosphatase is present in hamster bronchial epithelium and alveolar type II cells, but absent in hamster vascular endothelium and muscle. Glucose-6-phosphate dehydrogenase is only present in hamster bronchial epithelium, whereas LDH may be found in all four areas of the lung.

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In mouse lung, on the other hand, alkaline phosphatase is virtually absent from all cells. G6PD is found only in bronchial epithelial cells and LDH is noted in bronchial epithelium and alveolar type II cells but not seen in mouse connective tissue.

We would consequently expect to find G6PD and LDH enzymes in mouse epithelial cells but not in mouse fibroblast cells.

4. Samples of each type of cell line will be examined by electron microscopy (EM). These studies were originally performed by Dr. Ellen Dirksen at our Institute, but must be done subsequently under contract with the EM laboratory at the University. Although the presence of desmosomes is not definitive for epithelial cells (38), their presence and that of other morphologic structures can be suggestive. EM examination has been a helpful method for studying morphologic change in cultured cells.

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5. The cells will be plated on coverslips at low concentrations to provide distinct colonies. Cells will be labeled with ^{3}H-TdR to determine their growth characteristics. Epithelial-like colonies usually become labelled peripherally, while fibroblast colonies are equally labelled centrally and peripherally (Crocker and Levy, unpublished observations). This observation suggests that the growth of the epithelial-like cells is controlled by the phenomenon of contact inhibition. This technique may help in the identification of epithelial cell lines.

C. Sensitivity of the cell line to transformation

1. When an epithelial cell line is available, it will be used for studies of carcinogenesis.

a. It will be checked for aryl hydrocarbon hydroxylase (AHH) by the techniques of Nebert, et al. and others (40-42). (This work will be done in collaboration with Dr. Ronald Rasmussen at our Institute and Dr. Richard Kouri in Bethesda).

b. If the enzyme is present, the cells plated at 50% confluency in 60 mm plastic dishes will be treated with varying amounts of hydrocarbons and checked for the induction of transformed foci. Exposure to chemicals will be at first for 10 days and if successful, shorter periods shall be tried.

c. In attempts to look at the kinetics of chemical transformation, the cells will be studied similar to the method used by DiPaoli, et al.(21). Cells will be plated at 5 x 10^2/60 mm dish. After 24 hours they will be exposed to carcinogens dissolved in DMSO. After 48-72 hours or longer, if necessary, they will be washed twice and incubated for an additional 7-8 days. Colonies of transformed cells will be counted.

d. The interrelationship of virus and chemical in the transformation of the cells will be examined, particularly in reference to the initial studies in rodent cells by Freeman, et al., and others (5-12). Similar experiments using non-infected and chronically infected cells will be used. In the previous cases the transformed cells were fibroblasts.

e. The sensitivity of the epithelial cells to transformation by sarcoma viruses will be examined. It would be interesting to note if a sarcoma virus will give a different transformed cell morphology than a chemical, or chemical and leukemia virus together. Ikawa, et al. have suggested that the Kirsten murine sarcoma virus can transform Balb 3T3 cells and give rise to epithelial cell carcinomas (25).

D. Concerted Effect of Virus and Chemical on Transformation of Mouse Tissue Culture Cells.

As stated above, a line of NZB cells has been established which in its early passage maintains a normal epithelial-like morphology. On the addition of small amounts of hydrocarbons, areas of transformation appear which actually stand out from the dish-like "haystacks" (Fig. 6); these foci can be counted easily with the naked eye. We have determined that 3-MC can transform these cells after only 24 hours of treatment with concentrations of 0.5 µg/cc. Recent experiments have suggested that pretreatment of these cells with a non-carcinogenic hydrocarbon may induce high levels of AHH activity so that lower concentrations of hydrocarbons can now be detected by the presence of transformed foci. In this sense, the cell line is made more sensitive to the presence of potential hydrocarbon carcinogens.

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Dr. William Benedict in Los Angeles has also used these cells and finds effective transformation with cytosine arabinoside. We propose to continue our studies with the following projects in mind.

1. The cells will be pretreated with non-carcinogenic hydrocarbons and assayed for their possible increased susceptibility to transformation by small concentrations of potential carcinogens. In this manner, the line might be an excellent indicator of potential carcinogens in nature.

2. The carcinogenic potential of various fractions of tobacco smoke and smog air can be tested on the sensitized cells.

3. Since we know cytosine arabinoside induces transformation, we would like to try other antimetabolites such as hydroxyurea, 5-fluorodeoxyuridine and mitomycin (20). These agents work directly on DNA; their mechanism of action may be through chromosome breaks or induction of aneuploidy (44-46).

4. Dr. Sachs and his group have demonstrated a specific chromosome which is present in normal cells but absent after transformation (47,48). They refer to this chromosome as a "suppressor chromosome". In collaboration with Dr. Benedict in Los Angeles, who is examining the possible presence of this phenomenon in the murine system, we will be studying spontaneous transformants in this NZB cell line as well as those cells transformed by chemicals. It will be extremely interesting to note if a uniform absence of a chromosome does determine the malignant transformation of a cell. Similarly, the role various chemicals or antimetabolites may play to encourage the emergence of such a cell can be studied with this sensitive cloned NZB cell line.

5. This cell line, because of its sensitivity to chemicals, lends itself to the study of the timing of chemical transformation. We shall begin by examining the effect of cell synchronization on the degreee of transformation by a chemical. Next, we shall look at specific time periods after cell division to determine when the cell is most susceptible to transformation by a chemical. We shall expose the cell in culture for various time intervals following cell division in attempts to find the ideal time of the transformation event. In a related type of experiment, the cells in suspension will be exposed to chemicals at different concentrations. At various time periods, after incubation (1 h. up to 48 hrs.), aliquots of these cells will be removed, washed twice and plated at low cell density. The quantity of transformed colonies can then be determined. By this method, the length of time required for exposing to a chemical and the effective concentration of a chemical for transformation can be evaluated. Such information may help us understand the point of interaction between the cell's molecular makeup and the chemical.

E. Evaluation of C57 Epithelial-like Cultures

We have recently isolated an epithelial-like cell line from a C57/Bl mouse. This line has a typical "cobblestone" appearance and has the characteristics of contact inhibition (Fig. 7). Several clones of this line are now available. These C57 lines differ from the NZB cell line in that they have no detectable murine leukemia virus. On addition of carcinogenic hydrocarbons no foci of transformation have yet been detected. Toxicity of the chemicals has been demonstrated so we know these cells contain AHH activity.

Over the next year we propose to characterize these cell clones further:

1. We shall infect these cells with murine leukemia virus (MLV) and note if this virus alone can transform the cells after several passages.

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2. Cells chronically infected with MLV will be treated with carcinogenic hydrocarbons (particularly 3-methyl cholanthrene) and the combined effect of virus and chemical on transformation will be evaluated. It will be interesting to see if the MLV-infected C57 line acts like the cloned NZB line which has demonstrable endogenous "C" type virus (49-50).

3. We shall repeat the earlier experiments in which the cells were treated with chemical alone. In these new experiments the cells will be treated up to the level of toxicity. Surviving cells will be passed 10 times to determine any carcinogenic effect of the hydrocarbon on the C57 cells.

4. Cell lines will be infected with murine sarcoma virus and any foci formed will be isolated. These transformed cells and any resulting after chemical treatment will be inoculated into the brain of ALS treated NIH Swiss cells [in collaboration with Dr. Arnstein (51)] or into the lungs of recipient C57 mice (in collaboration with Dr. Furst). The type of tumor produced will be evaluated. It is hoped that transformation will produce carcinomas indicating these cells are of epithelial origin.

F. Establishment of Human Epithelial Cell Cultures

A special area for human cell cocultivation has been established for us at the Institute. We propose to begin the cultivation of human skin and tracheal cells using the techniques that are developed in these mouse experiments. These cultures would be available to test carcinogens on human cells. With certain RNA tumor viruses now available which grow in human cells, the possible concerted role of these viruses and chemicals in transformation can be examined. Preliminary experiments with human embryos, tracheas and lungs received from the Department of Pediatrics have indicated that these cells can be propagated.

Significance of the Research

Most human cancers are of epithelial cell origin. For this reason, it is extremely important to develop a model system for studying carcinoma induction. Tissue culture techniques when adapted to this system should offer the most inexpensive and efficient method for an in-depth examination of epithelial cell transformation. The murine system is best adapted to these studies since it is well-defined and its RNA tumor viruses have been well studied and can be detected by a variety of means. No successful cultivation of epithelial cells from mouse trachea and skin has been reported by any laboratory. We feel we have the most experience in these efforts and we would like to continue our attempts using other techniques both in tissue culture and in animals. Until we can develop the methodology for growing pure differentiated epithelial cells in vitro, the precise events leading to epithelial cell transformation in vivo cannot be defined. When a line of epithelial cells has been established, cellular transformation can be easily evaluated by tissue culture procedures which our laboratory has used in other studies of transformation by chemicals and/or viruses.

The work with the NZB and C57 lines will help answer questions about the nature of virus-chemical interactions. It appears that the presence in a cell of a virus (whether infectious or not) is necessary for efficient transformation by chemicals. The timing of the transformation event and the interaction of chemical, virus and host cell DNA can be approached by using these cloned cell lines. Once understood, the results obtained will be helpful for subsequent studies with established murine and human epithelial cell cultures. In summary, the studies presented in this proposal are aimed at answering the important question of how transformation of epithelial cells leading to carcinomas occurs in man.

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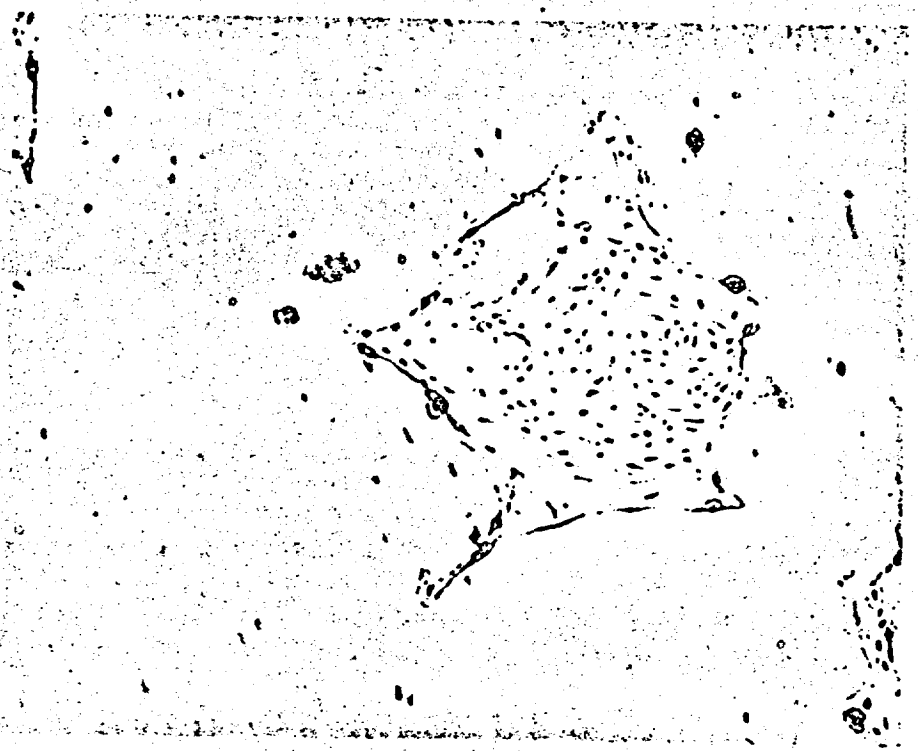

Figure 1

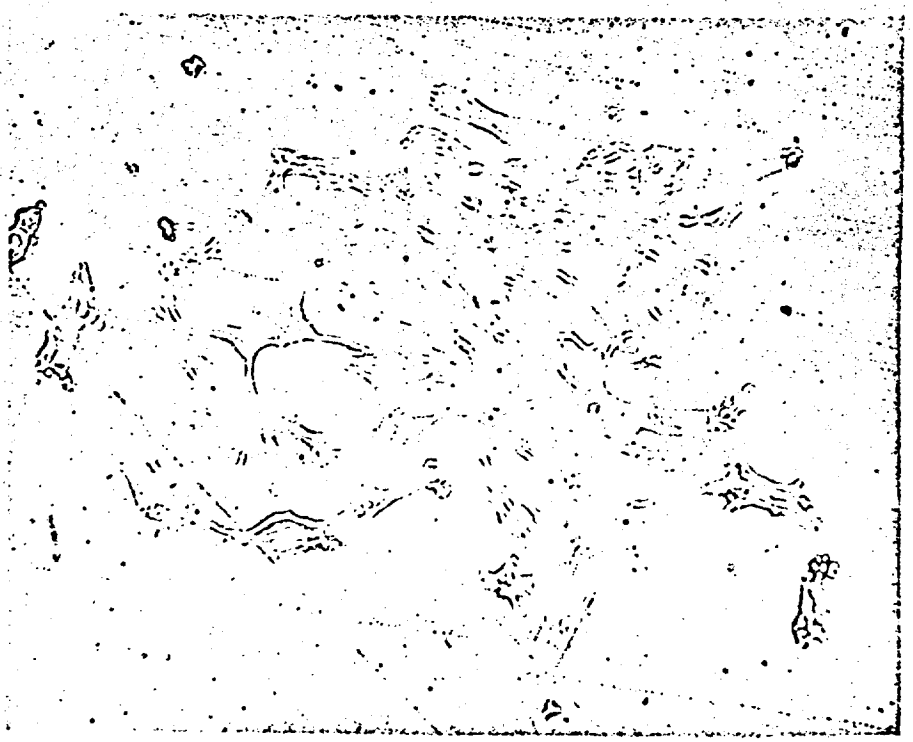

Figure 2

10035404M

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

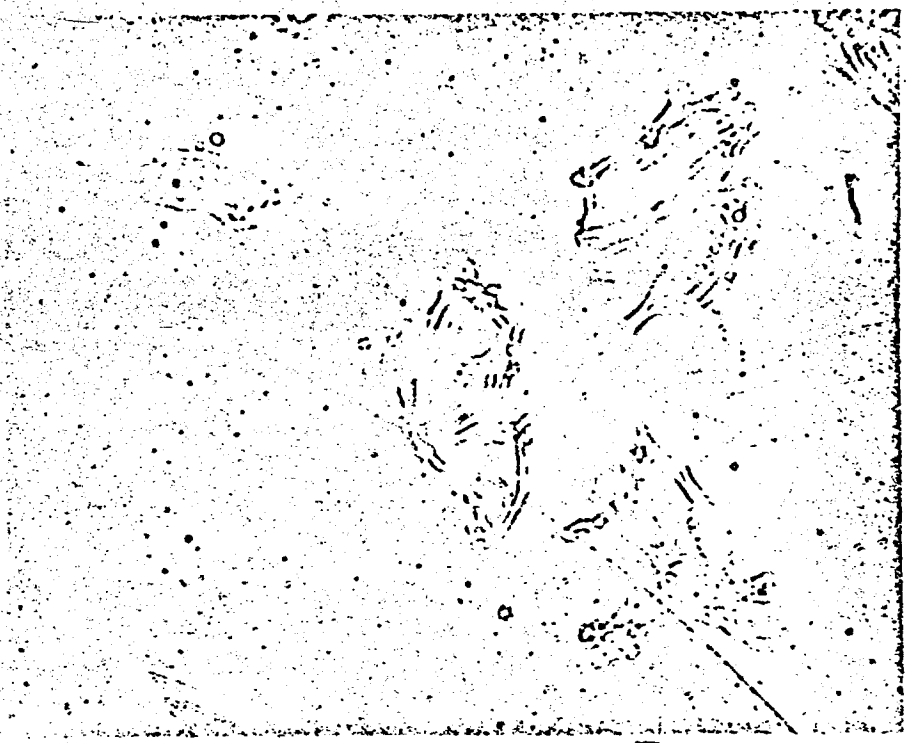

Figure 3

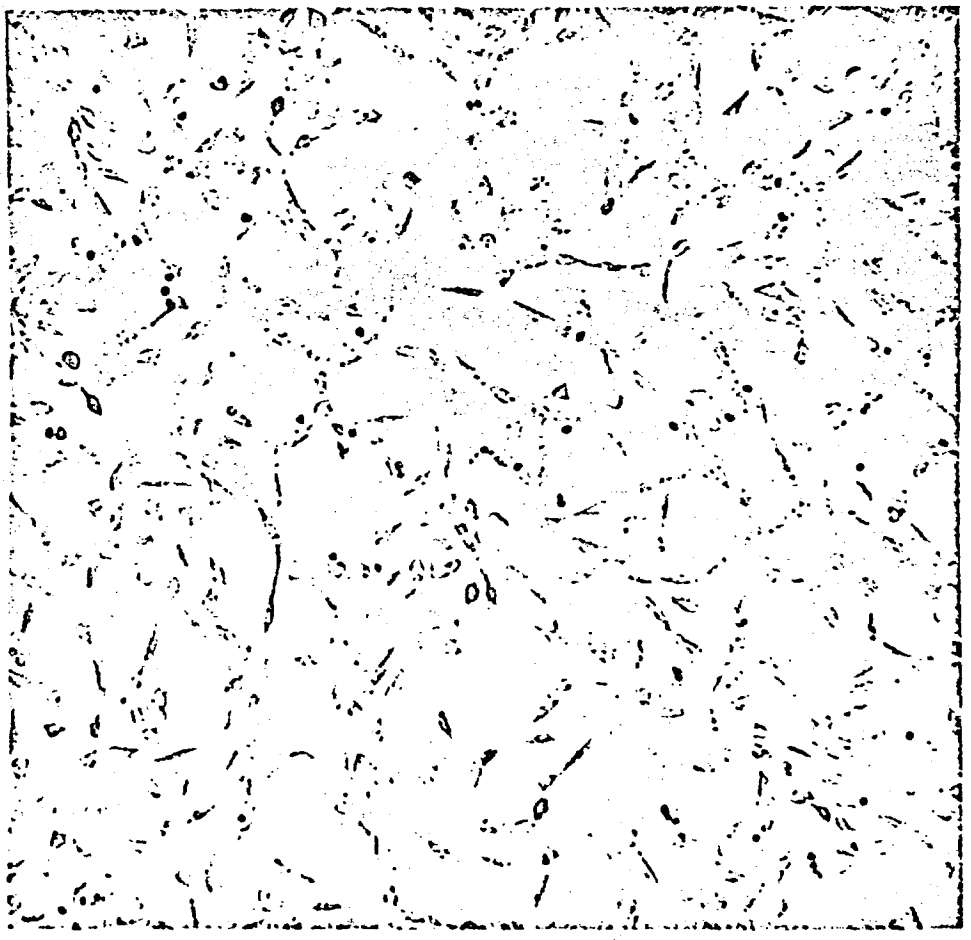

Figure 4

1003544022

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

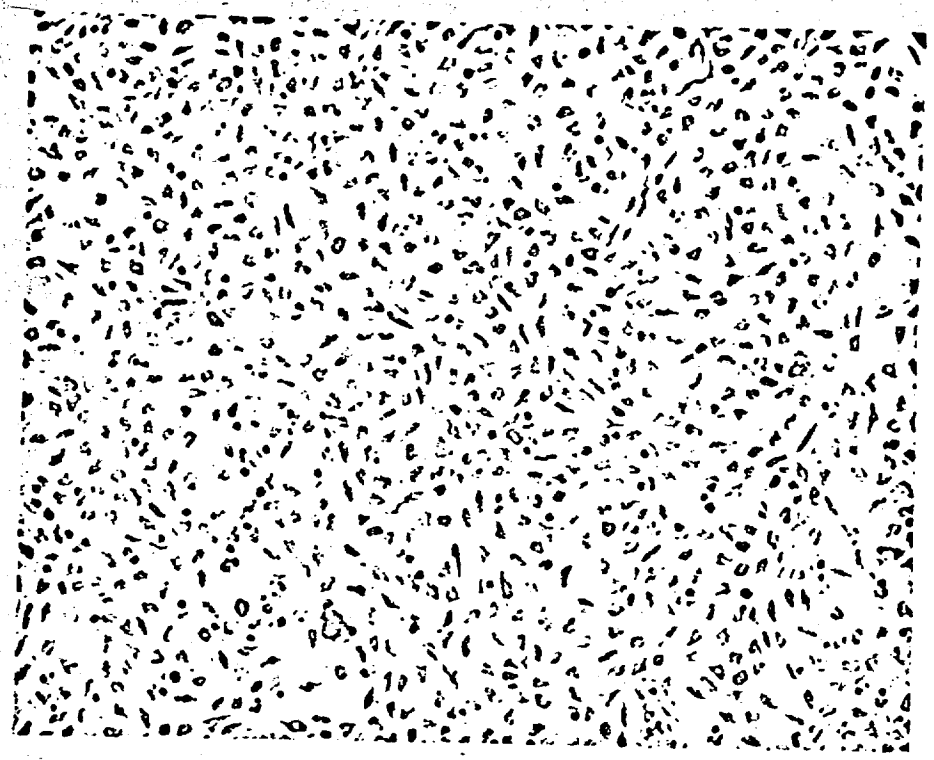

Figure 5

Figure 6

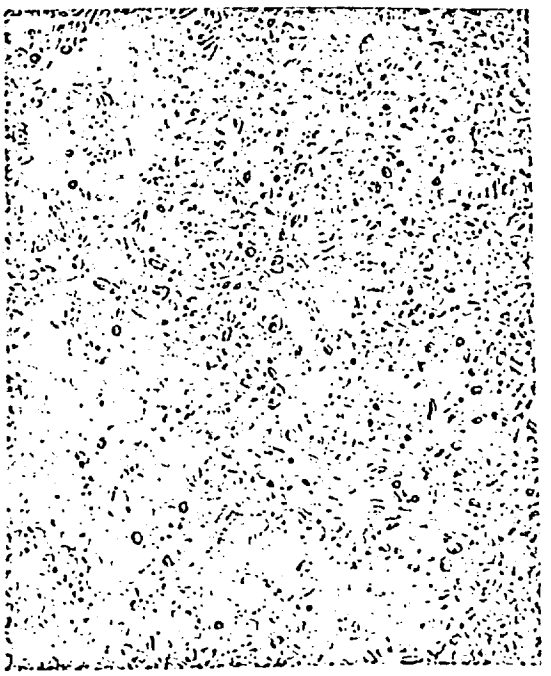

Figure 7

1003544073

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Legend to Figures

Fig. 1-3. Islands of epithelial-like cells recovered after trypsinization of adult BALB/c mouse tracheas .
Fig. 1: x40; Fig. 2: x60; Fig. 3: x60.

Fig. 4. A cloned line of NZB embryo cells established in continuous culture. Note the rectangular epithelioid morphology of the cells. x100.

Fig. 5. The same cloned NZB embryo cell line at confluency. x60.

Fig. 6. An area of cell transformation induced by 3-methylcholanthrene in the cloned NZB embryo cell line.

Fig. 7. A cloned epithelial-like cell line established from a C57/Black mouse. X60.

1003544074

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Room 1280 of the Cancer Research Institute, located in the Medical Sciences Building is used for the experimentation with animal cells. This laboratory has approximately 500 square feet with tissue culture units furnished with benches, essential outlets and disposal areas including an autoclave.

Inverted and dissecting microscopes and incubators are in the laboratory. An ultraviolet source for XC testing has been installed and refrigerators and liquid nitrogen tanks for storage of virus and cell lines are present.

A separate area for human cell cultivation has been established in Room 1273-S. It has a Baker 6000 Laminar flow hood, sink, and outlets for an incubator and autoclave. All precautions outlined in the NIH Biological Hazards monograph have been followed (see Appendix).

Additional facilities required:

None

10035440?5

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested) (including 15% fringe benefits)	% time	Amount
Technical		
Siegel, Susan, Staff Res. Assoc. II	100	$ 14,159
Ramirez, Michele, Secretary II	50	4,824
Clark, Vera, Lab Asst. I	50	4,068
Sub-Total for A		$ 23,051*
B. Consumable supplies (by major categories)		
Media and sera		7,500
Animals, purchase and maintenance		1,500
Glassware, pipettes, plastic flasks, petri dishes		2,500
CO_2 tanks and liquid N_2		900
Cells: mouse, rat, human		1,800
Sub-Total for B		14,200
C. Other expenses (itemize)		
EM examinations		2,000
Publication and photography		700
Travel		500
Sub-Total for C		3,200
Running Total of A + B + C		40,451
D. Permanent equipment (itemize)		
Sonicator, ultrasonic system, Biosonik III		950
CO_2 incubator (National)		1,650
Sub-Total for D		2,600
E. Indirect costs (15% of A+B+C)		6,068
Total request		49,119

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	24,204	14,910	3,360	--	6,371	48,845
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Justification of the Budget

Salaries:

Our studies with epithelial-like cell cultures have opened up several interesting areas for further research. The techniques for cultivation of epithelial cell clones and characterization of the cell types by animal inoculation and tissue culture testing require the help of a technician and a part-time laboratory worker. The latter's job includes the care of the animals and washing dishware since the Institute does not have a central kitchen.

Susan Siegel has been an integral part of our research team. She has been actively engaged in the cloning and propagation of mouse epithelial cells and the development of sensitive assays for chemical carcinogenesis. Vera Clark, besides keeping us equipped with sterile glassware, has helped in the removal of animal tracheas and skin for epithelial cell cultivation.

The Institute does not provide a secretary; Michele Ramirez has been with us for the past year and we need her for all the budgeting and secretarial tasks that this project entails.

Supplies and Equipment:

Since epithelial cells are very fastidious we must buy already prepared media. These are checked by us beforehand to be certain we have the best lots for maximal cell growth without any toxicity. This media is expensive, but is necessary for optimal results in these studies. Moreover, media, particularly for the sequential passage experiments cited in this proposal, must be specially ordered from Grand Island Biological.

We have not been able to obtain a sonicator for our CF testing. We would like to be able to do our own sonication so that we do not need to request this service from Dr. Huebner's staff. The incubator is needed for maintaining our cell lines.

Others:

The examination of our epithelial cells by electron microscopy has been one interesting method of detecting morphological differences and the presence of non-infectious virus particles. At this time we must pay a set price of $80 for each specimen examined. For $2,000 we can arrange a contract with a group here at the University who will examine all the mouse and tumor specimens we would like over the oncoming year.

10035407

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

understood that the investigator and institutional
ers in applying for a grant have read and accept
Council's "Statement of Policy Containing Conditions
Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Jay A. Levy, M.D.

Signature Jay A Levy Date 7-17-73

Telephone 415 666-4071
Area Code Number Extension

ks payable to

eg s of the University of California

ing address for checks

ccounting Department - 1550 HSE

niversity of California

an Francisco, California 94143

Responsible officer of institution

Typed Name Mr. Stanley C. Bateman

Title Contracts & Grants Officer

Signature _____ Date _____

Telephone 415 666-2977
Area Code Number Extension

10035440 78

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

APPENDIX

(Rolf Xerox)

Safety Procedures

1. Baker 6-foot Laminar Flow Hood will be used for experiments with human and cat material.

2. Autoclave will be in close vicinity to inactivate any virus-containing material before general disposal.

3. All virus contaminated glassware will be emersed in Chlorox while awaiting autoclaving.

4. No pipetting by mouth will be permitted in the laboratory.

5. A serum sample will be taken initially from the entire staff and other personnel sharing the rooms. Further samples will be taken and stored every 6 months.

6. All personnel will be supplied with a pair of tennis shoes or comfortable rubber soled shoes for use only in the laboratory. They will be left in the lab at the end of the day. All personnel will wear either a long white coat buttoned to the top or a cloth suit which slips over his day clothes. This will be changed once or twice a week. All personnel supplies will be kept out of the room.

7. No eating or smoking permitted in the lab. Drinking fountains will be the sole source of water for drinking by personnel.

8. No female technician who becomes pregnant will work in proximity to this virus research.

10035440 29

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

1003544080

#891R1 - NEURATH

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 26, 1973

Grant application #891R1
CANCER

TO: The committee comprising Drs. Gardner, Huebner, and Meier

SUBJECT: Dr. Georg B. Neurath, Microanalytical Laboratory, Hamburg, W. Germany
First renewal application No. 891R1
"Kinetics of the Nitrosamine Formation in Tobacco Smoke"

History

Grant #891, for the calendar year 1973, was awarded in the amount requested, $41,000. Two additional years "priority in competition" were voted.

Application #891R1 requests $44,850. The original estimate for this year was $35,000.

Documents Submitted (attached)

1. Application dated July 18, 1973.

2. Progress Report #1 January 1 - July 10, 1973.

Comment

Staff comment may follow in due course.

FWN

FWN:gh F.W.N.

1003540081

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

#891R1

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 25 1973

Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐

Date: July 18, 1973

1. Principal Investigator (give title and degrees):

Dr. Georg B. Neurath

2. Institution & address:

Microanalytical Laboratory
2000 H a m b u r g 56 - Hexentwiete 32 - West Germany

3. Department(s) where research will be done or collaboration provided:

4. Short title of study:

KINETICS OF THE NITROSAMINE FORMATION IN TOBACCO SMOKE

5. Proposed renewal date: January 1, 1974

6. How results to date have changed earlier specific research aims:

No changes - see report

7. How results to date have changed earlier working hypothesis:

No changes - see report

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

8. Any additional facilities now required? Describe briefly:

None

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

No changes

10. Append outline of experimental protocol for ensuing year.
11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

None

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12. Summary progress report (append in standard form as separate document, unless recently submitted).

Source: https://www.industrydocuments.ucsf.edu/docs/hrcm0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s) even if no salary requested)

	% time	Amount
Chemist	30	8,000
Chemical Engineer	100	16,700
Technician	25	2,000

Technical

Sub-Total for A $26,700

B. Consumable supplies (by major categories)

Chemicals	1,500
Glass	2,800
Small apparates	4,000
Standard gases	1,500
Others	1,000

Sub-Total for B $10,800

C. Other expenses (itemize)

Travel expenses	1,500

Sub-Total for C $1,500

Running Total of A + B + C $39,000

D. Permanent equipment (itemize)

None

Sub-Total for D 0

E. Indirect costs (15% of A+B+C) E $ 5,850

Total request $44,850

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dr. Georg B. Neurath

Signature [signature] Date July 16, 73

Telephone W. Germ. 040 81 60 40
Area Code Number Extension

Checks payable to

Dr. Georg B. Neurath

Mailing address for check:

Hexentwiete 32

2 Hamburg 56, West Germany

Responsible officer of institution

Typed Name

Title

Signature Date

Telephone
Area Code Number Extension

10035440S5

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

#925 - RAO

1003544086

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 27, 1973

Grant Application No. 925
CANCER

To: The committee comprising Drs. Andervont, Gardner, and Jacobson

Subject: L. G. S. Rao, Ph.D., Regional Steroid Laboratory, Glasgow, Scotland
New application No. 925
"Correlation of the Levels of Plasma Steroids with those in Urine of Lung Cancer Patients and Controls"

History

Dr. Rao has been applying to CTR, unsuccessfully, since 1970 on this general topic.

When he was last denied by SAB early in 1972, Dr. Hockett forwarded to him (anonymously) comments by SAB and criticisms that had come from an outside reviewer.

Application No. 925 requests 3,950 Pounds Sterling plus two additional years.

Documents Submitted (attached)

1. Letter dated 23rd July 1973 with application dated 18th July 1973.
2. C.V. of L. G. S. Rao.
3. Reprint "Prediction of 1-Year Survival . . ." by Rao, British J. Surg., 59 977-979, 1972.

Comment

Staff comments may follow.

F.W.N.

FWN:wg
Encls.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

#925

Comm.
Dr. Andervont
Dr. Gardner
Dr. Jacobson

CANCER

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 18 July, 1973.

1. Principal Investigator (give title and degrees): Dr. L.G.S. Rao, Ph.D.

RECEIVED
JUL 26 1973

2. Institution & address: Royal Infirmary, Glasgow, G4 0SF.

3. Department(s) where research will be done or collaboration provided: Department of Steroid Biochemistry.

4. Short title of study: Correlation of the levels of plasma steroids with those in urine of lung cancer patients and controls.

5. Proposed starting date: 1st October, 1973.

6. Estimated time to complete: Three Years.

7. Brief description of specific research aims: Previous work by the applicant has shown that lung cancer patients show some abnormalities which are rarely found in normal men in the urinary excretion of some steroids. These abnormalities have been found to be related to the length of survival of both inoperable and surgically treated lung cancer patients. Therefore, it is felt that these results might help to throw some light on the nature of this disease. However, the urinary steroids are the end products of metabolism and excretion of the steroids secreted by the adrenal cortex and the testes. Any interpretation of the urinary findings would be difficult unless the levels of these steroids and their precursors in blood are known. Blood levels of steroids are also essential for tracing the source of the urinary abnormalities.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

8. Brief statement of working hypothesis:

The urinary steroid excretion pattern which is abnormal in lung cancer patients reflects abnormalities in plasma steroid concentrations in these patients. The alternative hypothesis which has to be excluded is that the abnormalities in urine are attributable to the abnormalities in the excretion of these steroids by the kidney.

9. Details of experimental design and procedures (append extra pages as necessary)

The main abnormalities in steroid excretion in lung cancer patients are the low androsterone and high 17-hydroxycorticosteroids (17-OHCS), whereas aetiocholanolone is normal (Rao, 1970). The possible significance of these findings in the aetiology or pathogenesis of lung cancer has been discussed (Rao, 1972). The main purpose of the present proposals is to try to trace the source of these abnormalities. Thus, as a first step, it is proposed to measure these steroids or their precursors in blood plasma in a group of lung cancer patients and controls, and compare the steroid pattern in blood with that in urine.

The precursors of the 17-oxosteroids in blood are dehydroepiandrosterone (DHA) and its sulphate from the adrenal cortex, and testosterone from the testes. However, sulphates of DHA and androsterone are the precursors which are found in much larger amounts compared to other precursors of the 17-oxosteroids. The sulphates of DHA and androsterone will be measured by a gas-chromatographic method (De Moor and Heyns, 1966).

It would be very valuable to measure the other precursors of 17-oxosteroids; namely, testosterone and its metabolites in plasma. These steroids include 5α-dihydrotestosterone and androstenedione, but all these three steroids are found in much smaller amounts in blood than the sulphates of androsterone and DHA. Therefore, the former steroids will need more sensitive methods, such as radioimmunoassay. Such assays are in routine use in this laboratory and need chromatographic separation of the individual steroids before quantitation by radioimmunoassay.

The 17-OHCS are derived mainly from cortisol and cortisone secreted by the adrenal cortex, and can be measured as plasma 11-OHCS or 'cortisol' by fluorimetry. A routine method for the measurement of plasma cortisol by competitive protein binding is available in this laboratory, and this method will also be used in some samples as an additional check of the fluorimetric method. The urinary steroids will be measured as was done previously (Rao, 1970).

During the proposed study, it is felt that priority should be given for the determination of the 11-OHCS and the sulphates of androsterone and DHA, because it is known that these steroids are the main precursors of the steroids investigated before in urine. Thus, an early comparison would be possible between plasma and urinary levels. Part of the specimens will be stored at -20°C for the determination of the other steroids by radioimmunoassay as the second part of the study.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

9. Details of experimental design and procedures - CONTINUED:

It is proposed to investigate about 50 lung cancer patients and the same number of chest disease controls and of normal men. Blood and urine specimens would be collected from 100 consecutive patients attending the chest clinic of this Hospital and of other hospitals in this area before a knowledge of diagnosis is established. It is estimated that approximately 50 out of the 100 would have the diagnosis of lung cancer confirmed. Those found not to have lung cancer will be used as chest disease controls for the lung cancer patients. All the laboratory analyses will be carried out 'blind', without a knowledge of diagnosis. General biochemical data, such as liver and kidney function, and also clinical data, will be collected for each patient. It is estimated that the first part of the study would be completed in two years and the second part in one more year.

References:

De Moor and Heyns, W. (1966). In Androgens in Normal and Pathological Conditions. Excerpta Medica Foundation, International Congress Series 101, p.54.

Rao, L.G.S. (1970). Lancet, ii,441.

Rao, L.G.S. (1972). Brit. J. Surg. 59,977.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

All the equipment required for the proposed study, such as gas-chromatographs, liquid scintillation spectrometers, are already available in this Department.

Bench space is available for one Technician.

An excellent liaison with the chest physiciansand surgeons in this and other hospitals in this area has been established for the past five years, and it is extremely easy to obtain access to patients and controls.

11. Additional facilities required:

None - apart from the staff and other requirements detailed in the budget.

1003544091

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount (In Pounds Sterling)
Dr. L.G.S. Rao	10%	Nil
Technical		
One Technician	100%	£1,750
	Sub-Total for A	£1,750
B. Consumable supplies (by major categories)		
Chemicals, including radioactive isotopes		£250
Glassware and other consumables		£250
	Sub-Total for B	£500
C. Other expenses (itemize)		
Secretarial assistance		£500
Travelling expenses to hospitals, other than this Hospital and to attend scientific meetings in Britain		£250
	Sub-Total for C	£750
	Running Total of A + B + C	£3,000
D. Permanent equipment (itemize)		
1. One ... Single-Pen Recorder, to replace an old recorder used with the gas-chromatograph		£350
2. One ... Deep-Freeze Cabinet		£150
	Sub-Total for D	£500
E. Indirect costs (15% of A+B+C)	E	£450
	Total request	£3,950

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	£1,900	£500	£850	-	£500	£3,750
Year 3	£2,000	£500	£950	-	£550	£4,000

1003544092

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

16. Other sources of financial support: NONE..... (Currently Active/Pending or Planned).
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

t is understood that the investigator and institutional officers in applying for a grant have read and accept he Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name L.G.S. RAO

Signature L.G.S. Rao Date 17th July, 1973

Telephone 041-552 3535, Extension 391.
Area Code Number Extension

Checks payable to

The Secretary, Board of Management,
Glasgow Royal Infirmary.

Mailing address for checks

Dr. L.G.S. Rao, Department of Steroid
Biochemistry, Royal Infirmary, Glasgow, G4 0SF,
Scotland, U.K.

Responsible officer of institution

Typed Name DR. J.K. GRANT.

Title HEAD OF THE DEPARTMENT OF STEROID BIOCHEMISTRY.

Signature J.K. Grant Date 23rd July, 1973

Telephone 041-552 3535, Extension 368.
Area Code Number Extension

1003544093

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

R: REDACTED MATERIAL

CURRICULUM VITAE

Name: L.G.S. Rao.

(Laxmansandra Gundappa Shankara Rao).

Date and Place of Birth: REDACTED
REDACTED

Marital Status, etc.: R REDACTED

Qualifications:

1. B.Sc. Hons. (Biochemistry and Botany) - 1949.
   University of Mysore.

2. M.Sc. (Botany) - 1954.
   University of Mysore.

3. M.Sc. (Biochemistry) - 1962.
   University of London.

4. Ph.D. (Biochemistry) - 1966.
   University of Newcastle.

Experience:

1. Lecturer in Biology (1949 - 1953) - University of Travancore.

2. Research Student (September, 1954 - April, 1956) - Department of Biochemistry, Indian Institute of Science, Bangalore.

3. Research Fellow, Indian Council of Medical Research (April, 1956 - September, 1957) - Department of Biochemistry, All-India Institute of Mental Health, Bangalore. The work involved the determination of constituents of body-fluids, including cerebrospinal fluid, paper chromatography of urinary amino acids and their metabolites and agar-gel electrophoresis of serum proteins in chronic schizophrenic patients.

4./

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

4. Medical Laboratory Technician (April, 1958 - December, 1959) - Bow and Paddington Group Hospitals, London. Part of this time was spent in participation in research on the effect of androgens on nitrogen and electrolyte balance in patients with disorders of the kidney. I have been recognised as a qualified Medical Laboratory Technician by the Ministry of Health.

5. Hospital Biochemist, Basic Grade (January, 1960 - December, 1962) - Chelsea Hospital for Women, London, S.W.3. Determination of steroid hormones and metabolites in body fluids of patients with endocrine disorders.

6. Junior Research Associate (December, 1962 - December, 1963) - Department of Physiology, The Medical School, University of Newcastle. Research for Ph.D. Degree and teaching of Biochemistry to Science, Dental and Medical Students.

7. Senior Biochemist (April, 1966 - June, 1968) - Psychosomatic Research Unit, Southern General Hospital, Glasgow. Research into Lung Cancer, Heart Disease and Depressive Illness. I was in charge of the biochemistry laboratory and responsible for both the day-to-day running of the laboratory as well as planning the research programme.

8. Lecturer in Biochemistry (July, 1968 - September, 1972) - Department of Psychological Medicine, University of Glasgow, Southern General Hospital, Glasgow, S.W.1. Duties same as in 7.

9. Senior Biochemist (October, 1972 - to date) - Regional Steroid Laboratory, The Royal Infirmary, Glasgow, G4 0SF. Determination of steroid hormones in various disorders in patients of this and other hospitals in this Region.

100354095

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Publications:

1. Rao, L.G.S. & Taylor, W.
Sex and species differences in conjugate formation during the metabolism of (4-14C) progesterone in vitro. (1963). Biochem. J. 90,30P.

2. Rao, L.G.S. & Taylor, W.
Sex and species differences in conjugate formation during the metabolism of (4-14C) progesterone by liver homogenates. (1965). Biochem. J. 96,172.

3. Rao, L.G.S. & Taylor, W.
Effect of preincubation of homogenate of glucuronide formation during the metabolism of (4-14C) progesterone by male and female rat liver. (1965). Biochem. J. 96,61P.

4. Rao, L.G.S. & Taylor, W.
Glucuronide formation during the metabolism of (4-14C) progesterone by cat-liver homogenate. (1965). Biochem. J. 96,62P.

5. Kissen, D.M. & Rao, L.G.S.
Steroid excretion and personality in lung cancer. (1969). Ann. N.Y. Acad. Sci. 164,476.

6. Rao, L.G.S.
Urinary steroid excretion patterns after acute myocardial infarction. (1970). Lancet, ii,390.

7. Rao, L.G.S.
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PREDICTION OF 1-YEAR SURVIVAL AFTER RESECTION OF LUNG TUMOURS FROM THE PREOPERATIVE STEROID EXCRETION PATTERN

By L. G. S. RAO*

PSYCHOSOMATIC RESEARCH UNIT, DEPARTMENT OF PSYCHOLOGICAL MEDICINE, SOUTHERN GENERAL HOSPITAL, GLASGOW

SUMMARY

A prospective study of 50 patients with lung cancer who were treated by surgical resection of their tumour has shown that most of those who died within 1 year after resection had abnormalities in preoperative steroid excretion whereas most of those who are alive had normal steroid excretion. Of the 21 patients who died within 1 year, 5 died in hospital, mainly as a result of surgical complications, and were found to have an abnormally low androsterone to aetiocholanolone ratio and a high total 17-oxosteroid excretion. Those who were discharged alive and well from hospital but who died within 1 year as a result of metastasis to other organs had a low androsterone level in relation to aetiocholanolone and to total 17-hydroxycorticosteroids. Thus, it has been possible to identify by their preoperative steroid excretion 12 out of 14 patients who died within 1 year although they had had a successful resection.

ALTHOUGH radical surgery offers the best chance of survival for patients with lung cancer compared with other methods of treatment, nearly half of the surgically treated patients die within 1 year. Therefore a method of prediction of the prognosis might be useful for the management of these patients. If the technique of prediction has a biochemical basis it might provide clues to the pathogenesis of this disease and might even lead to a therapeutic application. Such a chemical method of predicting prognosis has already been shown to be effective for patients with inoperable lung cancer (Rao and Hewit, 1970). The present report deals with the prognosis of patients whose lung tumours have been resected by a lobectomy or pneumonectomy.

PATIENTS AND METHODS

Fifty male patients, aged 36–68 years (mean 55·7 years), who had undergone a resection of their lung tumour were followed up for a period of at least 1 year from the date of surgery; 27 had had a pneumonectomy and 23 a lobectomy. Two early morning specimens of urine had been collected for steroid assay about 3 days before surgery. Details of patients and laboratory methods have been described elsewhere (Rao, 1970).

RESULTS

Twenty-one patients died within 1 year after resection and, of these, 5 died in hospital mainly as a result of surgical complications such as haemoptysis, peripheral shock, or pulmonary embolism (group 1). Of the remaining 16 patients, 2 died of heart failure and kidney failure 4 and 7 months respectively after surgery and had no obvious evidence of metastases. The remaining 14 patients left hospital after surgery in a reasonable condition but died within 1 year mainly as a result of metastasis to the other lung or to other organs (group 2). Twenty-nine of the patients were alive and most of these were well 1 year after resection (group 3). Steroid excretion before resection was compared in the three groups and showed some striking differences (*Table I*). Among the patients who died within 1 year, those who died in hospital showed some differences in steroid excretion compared with those who left hospital alive and well; the former patients had a lower androsterone (A) to aetiocholanolone (E) ratio than the mean for group 2, and they also had higher 17-oxosteroid (KS) and 17-hydroxycorticosteroid (17-OHcs) excretion levels compared with those in groups 2 and 3. Patients in group 2 had significantly lower excretion of A and E compared with that in group 3. In group 2, 12 out of 14 patients had a 17-OHcs : A ratio of more than 10, whereas in group 3 only 5 out of 29 patients had such a high ratio. Thus most of the patients in group 2 could be identified by their abnormal steroid excretion (*Fig.* 1). Two patients in group 2 died of heart failure and kidney failure respectively and these did not have any marked abnormalities in steroid excretion.

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DISCUSSION

The results indicate that it is possible to identify using a chemical method, even before surgical treatment, those lung cancer patients who have a poor prognosis despite resection of their lung tumours. The significance of these findings could be considerable in the management of these patients if it can be established that their abnormalities are of aetiological significance and are not merely a reflection of the stage of the disease. The latter possibility arises because in patients with inoperable lung cancer the same steroid abnormalities are significantly related to the prognosis (Rao and Hewit, 1970). Thus, in these patients the greater the 17-OHcs : A ratio the shorter is the survival time. The present results show that this ratio is equally useful in predicting the 1-year survival in patients whose lung tumours have been surgically removed. Therefore, it is possible that the 17-OHcs : A ratio indicates the presence of metastases which were not clinically recognizable, and thus reflects only the stage of the disease. An alternative possibility in surgically treated patients is that a high 17-OHcs : A ratio indicates a greater susceptibility to metastasis after surgery. Previous work (Rao, 1970) has shown that removal of the lung tumour does not result in the disappearance of these steroid abnormalities and therefore they cannot be considered as being due to the presence of the lung tumours. It is not likely that these abnormalities are the effects of the clinically unrecognizable secondary tumours, since the removal of the primary tumour did not result in the decrease of the abnormalities. Therefore it appears that these abnormalities in lung cancer are the characteristics not of the tumour but rather of the host, which may be involved in the defence against the growth and spread of the tumour. Some indications are already available of the possible ways in which steroid abnormalities may be involved in the defence mechanisms of the host against the tumour. These can be considered under three main aspects.

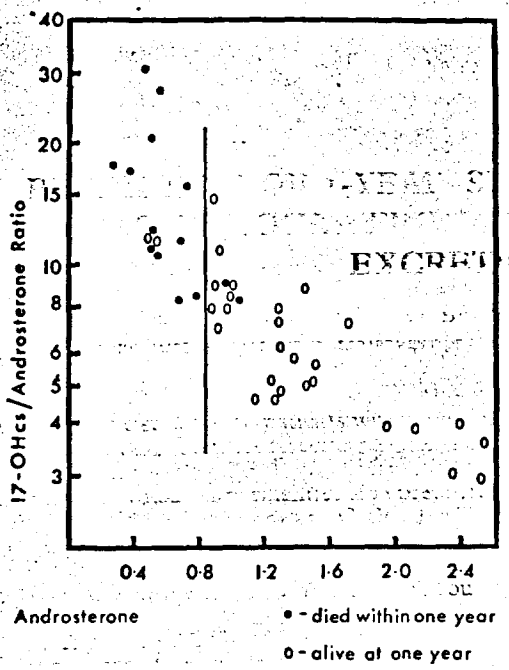

FIG. 1.—The survival of surgically treated patients.

1. Metabolic Effects.—Corticosteroids are mainly catabolic (Symington, 1969) and androsterone is anabolic (Furman and Howard, 1959). The high 17-OHcs : A ratio found in patients with a poor prognosis might shift the metabolic balance towards increased catabolism and lead to the cachexia found in many patients with a poor prognosis.

2. Haemostatic Effects.—It is known that cancer patients have an increased coagulability of their blood and this has been suggested as favouring the establishment of blood-borne metastasis (Thornes, 1967). Evidence for this view has come from the finding that the death-rate from cancer among persons receiving anticoagulant therapy is significantly lower than that in the general population (Thornes, 1967). It is known that corticosteroids increase coagulation by increasing the free fatty acids in the blood (Hoak and Robinson, 1963), whereas androsterone reduces coagulation by its ability to reduce platelet stickiness (Dufault, Tremblay, Nowaczynski, and Genest, 1961) and to increase fibrinolysis (Thornes, 1970). In fact, androsterone has been used prophylactically for this purpose in cardiovascular disease. Aetiocholanolone administration results in the increase of fibrinogen (Kimball, Vogel, Perry, and Wolff, 1967) and may thus enhance the tendency to coagulation. Thus, the low A : E ratio found in patients with a poor prognosis might have resulted in increased blood coagulation, favouring the establishment of blood-borne metastasis. Increased coagulability may also result in the formation of fatal pulmonary emboli.

3. Immunological Effects.—The importance of immunological mechanisms in the defence against cancer is now widely recognized (Editorial, *British*

Table I.—PREOPERATIVE STEROID EXCRETION IN GROUPS 1, 2, AND 3

GROUP	NO. OF PATIENTS	STEROID EXCRETION (mg. per g. creatinine per litre)			
		A	E	KS	17-OHcs
1	5	1·29 ± 0·46	2·90 ± 0·16*	7·02 ± 0·77	10·39 ± 3·56
2	16	0·72 ± 0·31*	1·19 ± 0·46*	4·18 ± 1·44*	8·26 ± 2·71
3	29	1·42 ± 0·66	2·11 ± 1·15	5·83 ± 1·93	8·11 ± 2·29

A, Androsterone; E, aetiocholanolone; KS, 17-oxosteroids; 17-OHcs, hydroxycorticosteroid.
* Significantly different from the mean of group 3 ($P < 0.01$).

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Medical Journal, 1970). The immunosuppressive effects of corticosteroids are well known and these steroids are used extensively for this purpose in tissue transplantation. It is likely that androsterone may enhance the immune response because of its anabolic effects. Thus, the high 17-OHcs : A ratio seen in patients with a poor prognosis may lead to a reduced immune response against the tumour.

It must be emphasized that although the above mechanisms are discussed separately two or more of these may be operating in unison to enhance the effect of steroid abnormalities. A careful investigation of these mechanisms in the prognosis of cancer would be a rewarding study.

A convincing method of proving that the steroid abnormalities in lung cancer are of aetiological significance would be to study the effects of correction of these abnormalities on the prognosis of these patients. Since the abnormalities in steroid excretion centre around a deficiency of androsterone these could be corrected by the administration of this steroid or a suitable precursor. Testosterone would not be a suitable steroid because the A : E ratio is not altered by its administration (Johnsen, 1968). Dehydroepiandrosterone also would not be a suitable precursor because this steroid is converted mainly to aetiocholanolone and thus would increase the abnormality found in lung cancer (Kirschner and Lipsett, 1964). It may therefore be necessary to administer androsterone or some other 5-alpha-androstane compound. Since the correction of the deficiency of androsterone could be carried out in conjunction with the existing methods of therapy it should be entirely feasible to conduct such a clinical trial.

Acknowledgements.—I thank Miss Margaret L. Hewit who carried out many of these steroid determinations with the assistance of Mr. G. Lynam and Mr. A. Clarke. I am grateful to Dr. R. J. Cuthbert, Dr. J. Cuthbert, Dr. J. T. Boyd, Mr. J. Hutcheson, Mr. M. A. Turner, Mr. R. A. McCluskie, and Mr. R. S. Barclay for allowing me to investigate their patients; the nursing staff for collecting specimens; and Professor T. Ferguson Rodger for his encouragement and interest. The Medical Research Council and Tobacco Research Council provided financial assistance.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 1, 1973

Grant Application No. 931
CANCER

To: The committee comprising Drs. Andervont, Gardner, and Huebner

Subject: Gerald M. Rosen, Ph.D., Duke University Medical Center
New application No. 931
"The Neoplastic State: Study of the Effects of Irreversible Amino Acid Analogs"

History

This is a spontaneous application, with no known antecedents. Application No. 931 requests $12,157, plus one additional year.

Document Submitted

Attached is application dated July 23, 1973 (22 pages).

Comment

No publications have been sent, but we can quickly provide for you if you wish copies of any of the papers cited.

F.W.N.

FWN:wg
Encl.

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#1931

CANCER

Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
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Rebuttal

Application for Research Grant
(Use extra pages as needed)

Date: July 23, 1973

1. Principal Investigator (give title and degrees):

Gerald M. Rosen, Ph.D.; Asst. Professor of Pharmacology

2. Institution & address:

Duke University Medical Center
Durham, North Carolina 27710

3. Department(s) where research will be done or collaboration provided:

Depts. of Physiology & Pharmacology and Biochemistry

4. Short title of study:

The Neoplastic State: Study of the Effects of Irreversible Amino Acid Analogs.

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: December 31, 1975

7. Brief description of specific research aims:

The immediate objective of the proposed research is to first synthesize a series of 3-(p-methoxybenzyl) sydnones with attached alkylating agents since these compounds have been reported to have antineoplastic activity against mouse carcinoma 755. The first phase of the study will then be to test these compounds in vitro utilizing the cell strains presently available in the laboratory. These cells include a rapidly proliferating mammary adenocarcinoma C_3H 2712 tumor and a slow growing mammary adenocarcinoma C_3H BA tumor and a normal mammary gland. The effect of the compound on cellular proliferation, mitotic index, DNA, RNA, and protein synthesis will be used to determine the effect of the drug.

The ultimate purpose of the proposed research is to develop a series of drugs with a potential to selectively destroy neoplastic cells with a minimum of damage to the function of normal cells. Towards this goal, we have the potential utilizing the talents and facilities of both biochemistry and Pharmacology Departments to coordinate information obtained from chemical, biochemical, and pharmacological studies on the effect of these agents in their interaction with macromolecular structures such as DNA and their effect on the synthesis and structure of essential cellular proteins. The potential of this basic information is to provide a number of agents for chemotherapy.

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RESEARCH PLAN

A. Introduction:

1. a. Immediate Objective

The immediate objective of the proposed research is to first synthesize a series of 3-p-(methoxybenzyl) sydnones with attached alkylating agents since these compounds have been reported to have antineoplastic activity against mouse carcinoma 755. The first phase of the study will then be to test these compounds in vitro utilizing the cell strains presently available in the laboratory. These cells include a rapidly proliferating mammary adenocarcinoma C_3H 2712 tumor and a slow growing mammary adenocarcinoma C_3H BA tumor and a normal mammary gland. The effect of the compound on cellular proliferation, mitotic index, DNA, RNA, and protein synthesis will be used to determine the effect of the drug.

b. Ultimate Objectives

The ultimate purpose of the proposed research is to develop a series of drugs with a potential to selectively destroy neoplastic cells with a minimum of damage to the function of normal cells. Towards this goal, we have the potential utilizing the talents and facilities of both Biochemistry and Pharmacology Departments to coordinate information obtained from chemical, biochemical and pharmacological studies. The effect of these agents in their interaction with macromolecular structures such as DNA and their effect on the synthesis and structure of essential cellular proteins will be determined. The potential of this basic information is to provide a number of potential agents for chemotherapy.

2. Background:

a. Chemical

The use of alkylating nitrogen mustards as chemotherapeutic agents in arresting and in a few cases the elimination of cancer had its beginnings during the 2nd world war (1). Since that time, it is unfortunate, however, that only a few of the many thousands of such compounds synthesized through the years have proven to be efficacious (2). It has been proposed that at least part of this problem lies with the stability in situ, of the aziridinium ring. Thus Rosen and Ehrenpreis (3) have shown that the stability of the aziridinium ring is of utmost importance in determining the degree of irreversibility. In the case of nitrogen mustard-antineoplastic agents, for example, the potency of the agent is directly related to its ability to combine covalently, forming an irreversible bond, and thus alter the function of the cell. If the aziridinium ring is too

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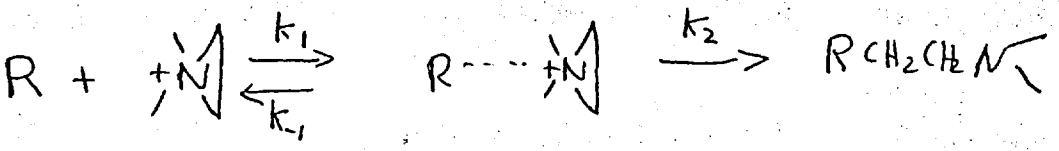
stable, reaction with tissue nucleophiles becomes difficult or impossible and the desired specific irreversible effects of the drug are not realized. By the same token, if the aziridinium ring is insufficiently stabilized, it is too rapidly hydrolyzed to the 2-hydroxyethylamine and the drug will again act only as a reversible agent. Such an explanation has been used to explain the in vivo and in vitro potency of dibenamine and phenoxybenzamine (3) and could readily substantiate similar results with nitrogen mustards. Rosen et al. (3,4,5) have suggested that the aziridinium-aromatic interacting distance has to be sufficient as to allow the aziridinium ring to be stable long enough to permit the drug to diffuse, intact, to the site of action. An example of such a drug although not an antineoplastic agent is the irreversible local anesthetic, 2-[2-chloroethyl)-methylamino]ethyl 4-ethoxybenzoate, whose aziridinium ring has a half life at 30° of 83 minutes (5).

Unfortunately, the lack of specificity of nitrogen mustards has been a major drawback with the use of these agents in cancer chemotherapy. One aspect of this problem as stated above is the stability of the aziridinium ring as compared to its alkylating ability. Furthermore, we have found in our earlier work with local anesthetics, that specificity (in this case, specific alkylation of the receptor sites on the nerve membrane) is related to the ability of the compound to diffuse to the area of interest. Thus there are many factors that are critical in addition to the alkylating moiety to obtain specific pharmacological action. Such is the case of phenoxybenzamine and dibenamine which possess an aziridinium ring of sufficient stability to allow these compounds to alkylate the adrenergic receptor but these agents are without pharmacological effects (3) at the nicotinic receptor of the neuromuscular junction. This phenomenon of specificity has been further explained by the kinetics of irreversible bond formation by dibenamine (6) and local anesthetics (7). Initially, a reversible electrostatic complex is formed between the receptor and the new drug, with time this equilibrium gives way to a non-competitive block, which cannot be reversed within a kinetically measurable time period.



Without this transport concept, indiscriminate alkylation increases to such a degree that statistical distribution of the drug is observed. We propose to overcome much of the problem of the lack of specificity by preparing an agent that not only contains an antitumor moiety but also has a sufficiently stable alkylating pharmacophore. This series of compounds will be discussed in depth later.

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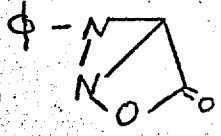
Earl and Mackney (8), working at the University of Sydney, found that treating N-nitroso-N-phenylglycine with acetic anhydride gave a fused ring (I) which they called "sydnone". In 1949, Baker et al, (9) found that the term fused ring was misleading and suggested that structure (II) was more appliable. At the same time Simpson (10) determined that "sydnone" was a charged species although totally neutral. Since the late 1940's, much work has been done on this unusual ring system, with the determination that a class of compounds called "sydnones" is aromatic in nature. The earliest reported consideration of sydnones as biologically important agents was made by Brookes and Walker (11), when they prepared several 3-methyl-4-alkyl-sydnones as potential amino acid antagonists. A number of sydnones have been examined for possible antitumor activity (12). Greco et al. (13) reported activity against carcinoma 755 in mice with 3-(p-methoxybenzyl) sydnone. Under the right specific circumstances the potential use of sydnones as antineoplastic agents, may be enhanced by their similarity in structure to amino acids.



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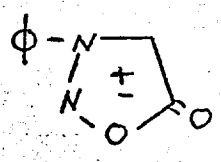

II

b. Biochemical Review of Alkylating Agents

The alkylating agents were the first group of synthetic compounds recognized for their anti-cancer properties. During World War I the bone marrow depressant effects of sulfa mustard bis (β-chlorethyl) sulfide, was noted but ignored. The revival of investigation in potential was gases during the Second World War included among other compounds the nitrogen mustards. In these studies the bone marrow depressant action of one of these agents, tris, (β-chlorethyl) amine hydrochloride suggested its potential value in the treatment of neoplastic diseases of the blood forming organs. The first clinical studies were begun by Gilman in 1942 (Science 103, 656, 1946). In fact, Rhoads summarized clinical experiences of 160 patients with neoplastic diseases in 1946 (JAMA 131, 656, 1946).

The original derivation of the term alkylating comes from the word alkane and has been defined by Warwick (Cancer Research 23, 1315, 1963) as "those compounds capable of replacing a hydrogen atom in another molecule by an alkyl radical".

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All of the alkylating agents are highly reactive chemical compounds capable of reacting with nucleophilic groups such as amino or sulfhydryl groups. Some of the alkylating agents are so chemically active that they will react with water within a few minutes. For this reason many of the alkylating agents must be stored as dry powder and dissolved immediately before administration.

There are two types of alkylating agents: monofunctional and biofunctional. The monofunctional alkylating agents which have only one active alkylating group are far less active in their anti-tumor activity than those containing two alkylating groups. The increased reactivity of bifunctional alkylating agents might have its explanation in that alkylating agents act by binding to DNA (Lawley and Brookes, Nature 206, 480, 1965).

The evidence that alkylating agents may have their primary function in the interstrand binding of double stranded DNA molecules is strengthened by the following observations:

1) Alkylating agents are both mutogenic and carcinogenic.

2) Both in vivo and in vitro alkylating agents produce fragmentation and clumping of chromosomes.

3) Alkylating agents inactivate DNA containing viruses much more readily than RNA viruses.

4) Alkylating agents do not inhibit bacteriophage containing a single stranded DNA although they are effective in alkylating DNA of the host which is double stranded DNA.

5) Alkylating agents are relatively ineffective inhibitors of protein function although they readily inhibit mitosis.

6) There is evidence of interstrand binding from the N7 in the guanine of one strand to the N7 of guanine in the opposite strand and that this interstrand binding prevents the separation of the two strands of DNA in the double coiled helix that is necessary for cell replication.

7) di (guanine-7-yl) derivatives of sulfa mustards have been isolated when this agent is reacted with DNA. Thus these observations would explain why the monofunctional alkylating agents are less effective than bifunctional agents.

It is on the basis of these observations that one could conclude that the efficacy of the alkylating agent is dependent

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on 1) reactivity of the alkylating groups as stated in our proposal and 2) the nature of the side chain that can be specifically designed to interact with the DNA molecule. The fact that alkylating agents are also mutogenic lends convincing support to their primary action at the level of the DNA molecule, as may be seen in recent reviews on alkylating agents: (Cline, Cancer Chemotherapy, Saunders Corp. 1971); (Clark, Cumley, McCay, and Copeland, Oncology, 1970, vol. 2, Experimental Cancer Chemotherapy, Yearbook, Medical publishers, 1970).

3. Rationale

As suggested in the Objective section, the study of the mode of action of sydnone nitrogen mustards on cancerogenic cells by means of refined pharmacological, biochemical and chemical techniques leads to an integrated concept of the characteristics of the neoplastic state.

As stated in the Background section, few of the many thousand nitrogen mustard compounds have been useful as antineoplastic agents. This is due in part to the variable stability of the, in situ, aziridinium ring. In addition, there appears to be no significant pharmacological structure that one can correlate to activity. It has been known that planar molecules might be able to "fit" into the DNA macromolecular structure and thus alter the programming process. The choice of sydnone nitrogen mustards as possible therapeutic agents is based on several pieces of evidence: Firstly, the sydnone ring is planar and in addition it has been used as amino acid analogs. The fact that the sydnone is planar would increase its capacity for intercalation into DNA and in addition as an amino analogue it has the potential to augment its transport. Secondly, Rosen et al, (3,4,5) have determined a possible aromatic-aziridinium distance that allows the ring to be sufficiently stable so that indescriminate aklylation is diminished greatly, which leads to a decrease in toxicity. By the techniques discussed in the Methods of Procedure section, known biochemical techniques can determine the site of action of these drugs.

B. Specific Aims:

Our immediate objective is to determine the effect of sydnone nitrogen mustards on normal and malignant cells in vivo and in vitro.

In the event of their efficacy, we will apply for another grant broadening out studies.

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C. Methods of Procedure:

a. Biochemical Studies

The initial biochemical investigations of the alkylating agents will first include a determination of the LD50 utilizing both C57 and C3H mice from Bar Harbor Jackson Memorial Laboratories. Two types of tumors will be studied in these mice. One represents a fastgrowing spontaneous adenocarcinoma H2712 (Dunham, L. C. and Steward, H. L., 1953, J. of the National Cancer Inst. 13, 1299). This tumor is usually palpable within three days, can be transplanted in ten days and kills the host between 9 and 16 days, and is therefore considered a very fastgrowing tumor. The slowgrowing tumor C3HBA tumor (reference is same as for the H2712) is also a spontaneous tumor of the mammary gland in C3H/An mice. The tumor is usually palpable within six days, is transplantable within 10 days and kills the host in 3 to 7 weeks.

Cells from these animals have been established in tissue culture in Waymouth media MAB87/3 (Charity Waymouth, Cultivation of Cells and Chemically Defined Media and the Malignant Transformation of Cells in vitro, W. Junk, Publishers, The Hague 1965). Most of our studies will utilize both tissue and cell culture in that they are most amenable to study the effect of small quantities of the compound that would not be subjected to biochemical alterations frequently found in vivo. In addition to these cells normal chick embryo fibroblasts and transformed fibroblasts obtained from the laboratory of Dr. Ralph Smith will be used to compare normal euploid cells and cells that have been transformed by virus.

One of the readily identifiable characteristics of normal euploid cells is that represented by contact inhibition as first described by Eagle and later amplified by a large number of other investigators (Cancer Research 29, 1770, 1969). The characteristics of this contact inhibition represent an alteration in gene expression of the normal cell to inhibit the replication and the transcription of its DNA, the result of which is the cessation of cell division and motility of the normal cell population. In contrast, cell cultures obtained from tumors, or cells that have undergone transformations no longer exhibit this characteristic of contact inhibition. We propose then to utilize these characteristics as a preliminary identification of the alteration of a normal to a malignant culture in vitro, and to determine the effect of our synthesized compounds on their ability to regain the capacity of contact inhibition exhibited by normal cells.

All of our studies will include determination of total DNA followed by the method of Ceriotti, G. (J.B.C. 214, 59, 1955) as

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modofied by Short, et al., (Short, E., Warner, H. R. and Koerner, J., J.B.C. 243, 3342, 1968). DNA synthesis will be followed by tritiated thymidine uptake using the procedures in our laboratory and protein synthesis followed by the utilization of C^{14} labelled leucine and RNA synthesis followed using labelled uracyl. Total protein will be determined by the method of Lowry et al., (J.B.C., 193, 265, 1951). The original procedure will be used for the determination of RNA (Ceriotti, J.B.C., 214, 59, 1955).

It is reasonable to assume that an antineoplastic agent will either specifically interact with the cell membrane of the malignant cell and thereby alter its permeability and/or will interact with the DNA by intercalation and alkylation. It is also reasonable to assume that the intercalation into the DNA would require a planar ring and that alkylation could be accomplished by a nitrogen mustard type side chain.

Thus the intercalation model of Lerman (Lerman, L. F., J. Molecular Biology, 3, 18, 1961) has been widely accepted as the mode of strong (primary) interaction between acridines and DNA (Waring, J. Mol. Biol. 54, 247, 1970) and has led to a plausible hypothesis for the mechanism by which acridines produce frame shift mutants. Recently a great deal of evidence has also accumulated that the phenomenon of intercalation may extend to a much wider range of drugs than just the acridines. Thus in addition to carcinogens, tranquilizers and ballucinogens, a number of chemotherapeutic agents may also have their mode of action by intercalation (Waring, J. Mol. Biol. 54, 247, 1970).

As a diagnostic feature of the intercalation process, we will use the technique of drug induced local uncoiling of the double helix of a $\Phi\chi$147 RD DNA. In this system the uncoiling results in the removal and reversal of supercoils of closed circular DNA revealed by changes in sedimentation coefficient (Waring, J. Mol. Biol. 54, 247, 1970). It should also be pointed out that the conversion from a plectonemic form (supercoil) to its paronemic form (open circle) may also be accomplished by the introduction of a single nick in one of the strands (Waring, J. Mol. Biol. 54, 247, 1970). Thus, in addition we will isolate the DNA by phenol procedures (Methods in Enzymology 12, Part A, B, and D, 1968, 1969 and 1971) and fractionate the DNA on cesium chloride isopycnic gradients, (Methods in Enzymology, 12 and 21), and examine it on alkaline sucrose gradients to determine if nicks have resulted from the alkylating sydnone.

b. Preparation of Nitrogen Mustart Sydnones

For simplicity, I will use structures where appropriate in discussing the development of these compounds. The synthetic

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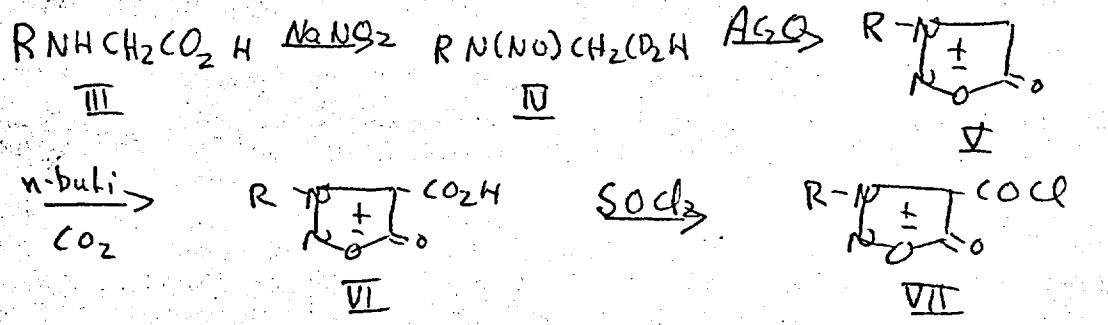
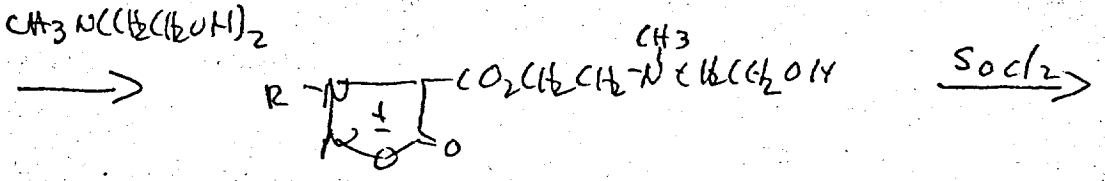
sequence used in the preparation of the sydnone ring system (V) is well documented in the papers of Earl and Mackney (8) and Greco, et al. (13). Greco, Pesce and Franco (14) prepared 3-phenylsydnone-4-carboxylic acid (where R = Phenyl, VI) by the reaction of 4-lithium-3-phenyl-sydnone with carbon dioxide. Conversion to the acid chloride (where R = Phenyl, VII) using thionyl chloride gives a white solid (15). The remaining steps in the development of the sydnone nitrogen (IX) mustard have been discussed by Rosen, et al. (5).

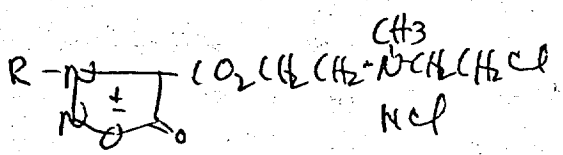


III IV V

VI VII



VIII



IX

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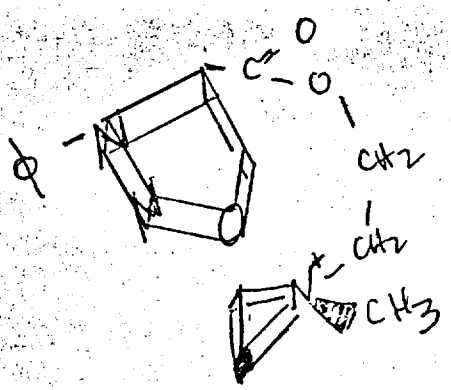
As suggested by the structure below, there is a possibility for the aziridinium ring to be stabilized against rapid hydrolysis by the interaction of the "banana bonding" electrons of the aziridinium ring and the aromatic electrons of the sydnone nucleus. This type of interaction has been suggested (16,5) to account for the increased stability of the aziridinium ring in the irreversible local anesthetic, 2-[chloroethyl)methylamino]ethyl 4-ethoxybenzoate.



D. Significance

There will be two immediate contributions from the proposed research: 1) The effect of sydnone-nitrogen mustards will be clarified. 2) By the use of this irreversible agent, our understanding of the biochemical processes underlying cancerogenic growth may be better understood. These accomplishments hopefully will be of significance in the following manner:

1) Identification of the site of action of sydnone nitrogen mustards would provide us with a better understanding of the mechanism of cancerogenic growth.

2) One or more of the compounds could prove of great value therapeutically for the purposes of arresting and/or eliminating cancer.

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References

1. A. Gilman and F. S. Philips, Science, 103, 409 (1946).

2. M. Ochoa and E. Hirschberg, In: Experimental Chemotherapy, Vol. 5, Ed. R. J. Schnitzer and F. Hawking, Academic Press, New York, N. Y. 1967, p. 1.

3. G. M. Rosen and S. Ehrenpreis, Trans. N.Y. Acad. Sci. 34 255 (1972).

4. G. M. Rosen, S. Ehrenpreis and A. Karoutsou, Arch. Int. Pharmacodyn. 203, 215 (1973).

5. G. M. Rosen, S. Ehrenpreis, T. W. Mittag and F. J. Stubbins, J. Med. Chem. 14, 514 (1971)

6. M. Nickerson and W. S. Gump, J. Pharmacol. Exptl. Therap. 97, 25 (1949).

7. G. M. Rosen and S. Ehrenpreis (Submitted to Nature).

8. J. C. Earl and A. W. Mackney, J. Chem. Soc. 899 (1935).

9. W. Baker, W. D. Ollis and V. D. Poole, J. Chem. Soc. 307 (1949).

10. J. C. E. Simpson, J. Chem. Soc. 94 (1946).

11. P. Brookes and J. Walker, J. Chem. Soc. 4409 (1957).

12. H. U. Daeniker and J. Druey, J. Hel. Chim. Acta, 40, 918 (1957).

13. C. V. Greco, W. H. Nyberg and C. C. Cheng, J. Med. Pharm. Chem. 5, 861 (1962).

14. C. V. Greco, M. Pesce and J. M. Franco, J. Heterocyclic Chem. 3, 391 (1966).

15. K. Kishimoto and M. Ohta, Nippon Kagaku Zasshi, 83, 833 (1962); Chem. Abstr. 59, 5152 (1963).

16. Y. Pittman, Jr. and C. O. Olah, J. Amer. Chem. Soc. 87, 2998 (1965).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

BIOCHEMISTRY:

Approximately 2,700 square feet of laboratory space is in use for the biochemical investigations in the laboratory of Dr. McCarty. Facilities include all heavy equipment required for the project, 2 Spinco ultracentrifuges, 2 amino acid analyzers, 2 Gilford Spectrophotometers, 1 packard Scintillation Spectrometer, 2 Sorvall centrifuges and continuous flow equipment.

PHARMACOLOGY:

Approximately 1,500 square feet of laboratory space is in use for the chemical and pharmacological investigations in the laboratory of Dr. Rosen, which does not include cold room, animal quarters, and secretarial offices. Facilities include equipment needed to synthesize and test the sydnones. The new equipment is needed to replace those instruments borrowed from Dr. T. Narahashi which include: a pH meter, two balances.

11. Additional facilities required:

The chemistry Department has a N.M.R. spectrometer which is available for my use.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

note additional sheets

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

BIOGRAPHICAL SKETCH

NAME: Gerald M. Rosen

TITLE: Assistant Professor of Pharmacology

BIRTH: REDACTED

EDUCATION: Lowell Technological Institute, B.S., 1965
Clarkson College of Technology, Ph.D., 1969

HONORS: N.I.H. Research Assistantship, 1965-1966.
Teaching Assistantship, 1966-1969.
Schering Fellow, 1967-1969.
New York State Incentive Scholar, 1965-1969.
Hoffman-LaRoche Postdoctoral Fellow with Professor S. Ehrenpreis, 1969-1970.
N.I.H. Postdoctoral Fellow with Professor S. Ehrenpreis, 1970-1971.
University Fellow, University of Zurich, Switzerland, with Professor P. G. Waser, 1971-1972.
N.I.H. Special Fellow to Duke University, 1973-1975.

REDACTED

RESEARCH INTERESTS: Neuropharmacology and Molecular Pharmacology.

PROFESSIONAL EXPERIENCE:

Teaching Assistant, Chemistry Department, Clarkson College of Technology, 1966-69.
Instructor, Department of Ophthalmology, New York Medical College, 1969-70.
Lecturer, Department of Biochemistry, New York Medical College, 1970-71.
Research Associate, The Institute for Medical Research and Studies, 1970-71.
Assistant (equivalent to Assistant Professor), Institute of Pharmacology, University of Zurich, 1971-72.
Assistant Professor, Department of Physiology and Pharmacology, Duke University Medical Center, 1972-

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PUBLICATIONS

F.D. Popp, J.M. Wefer, G. Rosen, A.C. Noble: Investigation of Philippine Plants for Alkaloids, Antimalarial Agents and Antineoplastic Agents, J. Pharm. Sci. 56, 1195 (1967).

F.D. Popp, J.M. Wefer, D.P. Chakraborty, G. Rosen, and A.C. Casey: Investigation of African Plants for Alkaloids, Antimalarial Agents, and Antineoplastic Agents, Planta Medica 343 (1968).

G. Rosen and F.D. Popp: Diazepines. V. The Reaction of Homophthalic Anhydride with Hydrazine. J. Heterocyclic Chem. 6, 9 (1969).

G. Rosen and F.D. Popp: Reaction of 6,7-Dimethoxy-3-isochromanone with Hydrazine. Can. J. Chem. 47, 864 (1969).

G. M. Rosen, F.D. Popp and F.Q. Gemmill, Jr.: 2-Benzyl-1,3,4-oxadiazolin-5-one and Related Compounds. J. Heterocyclic Chem. 8, 659 (1971).

G.M. Rosen, S. Ehrenpreis, T.W. Mittag and J.F. Stubbins: Physical and Pharmacological Properties of an Irreversible Local Anesthetic. J. Med. Chem. 14, 514 (1971).

G.M. Rosen and S. Ehrenpreis: NMR and Pharmacological Properties of a Series of Beta-haloethylamine Local Anesthetics. Fed. Proc. 30, 629 (1971).

S. Ehrenpreis and G.M. Rosen: The Irreversible Neuromuscular Blocking Action of Beta-haloethylamine Local Anesthetics: Site of Action, Temperature Dependence and Effect of Calcium. The Pharmacologist, 12, 265 (1971).

G.M. Rosen and S. Ehrenpreis: Physical and Pharmacological Properties of a Series of Ultra-Long Acting Local Anesthetics and Neuromuscular Blocking Agents. Trans. N.Y. Acad. Sci. 34, 255 (1972).

G.M. Rosen, S. Ehrenpreis and A. Karoutsou: Neuromuscular Blocking Activity of a Series of Beta-haloethylamines. Arch. Int. Pharmacodyn. 203, 215 (1973).

G.M. Rosen and S. Ehrenpreis: Neuromuscular Blocking Action of an Alkylating Local Anesthetic: Site of Action and Effects of Temperature and Calcium (in press).

P.G. Waser, A. Hoffmann, M. Schaub, W. Hopff, G. Rosen, and A. Chang: Affinity Labelling of Cholinergic Receptors with Curarazing and Depolarizing Drugs. Int. Congress Pharm. 5, 113 (1972).

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R: REDACTED MATERIAL

CURRICULUM VITAE

Name and Title: McCarty, Kenneth Scott, Ph.D.
Professor of Biochemistry

Address: Duke University Medical Center
Durham, N.C. 27710
REDACTED

Academic Discipline: Biochemistry of Differentiation and Control Mechanisms of the Metabolism and Synthesis of RNA and DNA

Parents: REDACTED

Place and Date of Birth: REDACTED

Marriage: REDACTED

Education:

B.S. Georgetown University, 1944
Ph.D. Columbia University College of Phys. and Surgeons, Dept. of Biochemistry 1958

Positions:

Research Biochemist, Food and Drug Adm., Washington, D.C. 1944-45
First Asst. to Director of Cancer Research, Lillia Babbit Hyde Foundation, Interchem, N.Y. 1945-52
Consultant Electron Microscopist, VA Hospital N.Y. 1949-54
Senior Staff Member of Tissue Culture Course, Cooperstown, N.Y. 1954
Instructor Biochemistry, Columbia University College of Physicians and Surgeons 1957-59
Consultant to Organon Corp., East Orange, N.J. 1956-60
Consultant to Parke Davis, Ann Arbor, Mich. 1960-61
Consultant to Microbiological Associates, Inc., Washington, D.C. 1962-68
Director of Graduate Studies 1967 to present
Associate Professor of Biochemistry, Duke University 1959-68
Professor of Biochemistry, Duke University 1968-

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R: REDACTED MATERIAL

Senior Staff Member (1) Research Training Program, Duke Medical School (2) Teaching Biochemistry for Medical Students (3) Graduate School, Department of Biochemistry (4) Director of Graduate Studies, Dept. of Biochemistry 1967 to present
Member of Research Council Duke 1970, Chairman for Medical School, 1971

Honorary Position:

Chairman of the Duke Membership Committee for the Southeastern Section of the Society of Experimental Biology and Medicine, 1960-70
Secretary of the Southeastern Section of the Society Exptl. Biol. and Medicine 1961-62, Secretary to New York Society of Electron Micr scopists, Executive Board Director of NYSEM, Vice Chairman Southeastern Section of Soc. Exptl. Biology and Medicine, 1963. Member of Evaluation Committee for Association of American Medical Colleges, 1970-71, Duke representative for Statesman in Medicine for Dr. B. Woodhall 1971.

Societies:

REDACTED

Honorary Fraternities:

REDACTED

Achievements of general interest:

Molecular Biology - Controls of RNA transcription and transport. Developed a procedure for growing mammalian cells under homeostatic conditions using the McCarty Homeostatic Cytogenerator. Developed methods of cell fractionation using the McCarty Cytodisintegrator and specially designed ultracentrifuge. Developed an Osteogenerator for growing organ cultures. Studied the biochemistry of pleuropneumonia like organisms in cell cultures. Investigated the amino acid metabolism of established cell strains. Studied the mechanism of synthesis of messenger RNA. Mechanism of transport of mRNA from the nucleus to the cytoplasm. Synthesis and activity of nuclear ribosomes. Hormone induction of enzyme activity in chick embryo duodenal cultures. Control of protein translation at the ribosome level in the sea urchin egg.

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Areas of Research and Specialized Knowledge:	Biochemical Investigations of Homeostatic Cell Cultures. Comparison of Biochemical Events with Ultrastructural Morphology (Electron Microscopy). Amino Acid Analysis of Cell Organ Cultures. In vitro Protein Synthesis, Histone fractionation, acetylation and phosphorylation. Hormone induction of histone modification
Contributor:	Experimental Medicine and Surgery, Cancer Research, Science Annals of N.Y. Academy of Sciences, Proc. of Soc. Exp. Biol. and Medicine, American Journal of Pathology, Experimental Cell Research, Excerpta Medica, Journal of Biological Chemistry, Archives of Biochemistry and Biophysics, Analytical Biochemistry, Biochimica Biophysica Acta
Special Grants or Fellowships:	Damon Runyon Memorial Fund 1958 American Cancer Society 1964- National Science Foundation 1959- Center for Study of Aging 1959- National Institutes of Health 1960- N.C. Heart 1969- Orthopaedics Res. 1969-
Special Positions:	1971 - Chairman of Research Council Representing the Medical School, Member of the Curriculum Committee, and Chairman of Undergraduate Education for Medical Students. Medical Student Advisor, Medical Center Curriculum Committee for First and Third Years. Program Director in Molecular and Cellular Aspects of Differentiation.
Special Contributions to:	1. "The Life Sciences - Recent Progress and Applications to Human Affairs, the World of Biological Research - Requirements for the Future". Edited by the Committee on Research in the Life Sciences published by the National Academy of Sciences 1970. 2. "Biology and the Future of Man" by Philip Handler. Oxford University Press 1970, Biological Sciences and the Advancement of Medicine 3. Editor of the Journal of the Tissue Culture Association "In Vitro". 4. Reviewer for journals, as Science, Annals of Internal Medicine etc.

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PERSONAL PUBLICATIONS

1973

McCarty, K.S., Jr., Jones, R., and McCarty, K.S., Sr., Hormone Effects on the Ultrastructure of Mammary Tumor Cultures, Abstract TCA Meeting June 1973.

Nixon,J., and McCarty, K.S., A Partial Characterization of Phosphorylated Acidic Nuclear Proteins, Abstract TCA Meeting June 1973.

McCarty, K.S., Sr., Jones, R., Nixon, J., and McCarty, K.S., Jr. Hormone Induction of Phosphorylation of Chromosomal Proteins in Normal and Malignant Mammary Glands in Culture, Abstract FASEB Meeting April 1973.

Nixon, J., McCarty, K.S., Jr., and McCarty, K.S., Sr., The Use of a Reorienting Density Gradient Rotor with Continuous Sample Flow for the Isolation of Calf Thymus Nuclei, Submitted Analy. Biochem., Jan. 1973.

Miller, D.M., Williams, R., and McCarty, K.S., Localization and In Vitro Specificity of Histone Acetylation, Biochim. Biophys. Acta, Submitted, 1973.

Miller, D.M., and McCarty, K.S., Specificity of Acetylation of Histone F2A1 in Normal and Leukemic Lymphocytes and Other Eukaryotic Tissues, Submitted Cancer Research, 1973.

Miller, R.D., and McCarty, K.S., The Histones of the Clear-Nosed Skate, Raja eglanteria, in preparation.

Miller, R.D., and McCarty, K.S., The Histones of the Spectaled Caiman, Caimon sclerops, in preparation.

Sanders, L.A., Schechter, N.M., and McCarty, K.S., A Comparative Study of Histone Acetylation, Histone Deacetylation, and RNA Synthesis in Avian Reticulocytes and Erythrocytes, Biochemistry, 12, 783, 1973.

1972

Marzluff, W.F., Sanders, L.A., Miller, D.M., and McCarty, K.S., Two Chemically and Metabolically Distinct Forms of Calf Thymus Histone F3, J. Biol. Chem. 247, 2026, 1972.

Marzluff, W.F., and McCarty, K.S., Structural Studies of Calf Thymus F3 Histone. I. Occurrence of Cysteine, Phosphoserine and ε-N-Acetyllysine in Cyanogen Bromide Peptides, Biochemistry 11, 2672, 1972.

Marzluff, W.F., and McCarty, K.S., Structural Studies of Calf Thymus F3 Histone. II. Occurrence of Phosphoserine and ε-N-Acetyllysine in Thermolysin Peptides, Biochemistry 11, 2677, 1972.

Hopkins, R.A., McCarty, K.S., and Allen, B.L., Serum B-Glucuronidase in Mice Bearing Fibrosarcomas, Orthopaedic Surgery Papers of the Duke University Medical Center and Affiliated Hospitals, Aug. 25, 1972 (Piedmont Orthopaedic Society).

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1972 (continued)

Sanders, L.A., Marzluff, W.F., Miller, D.M., and McCarty, K.S., Two Chemically and Metabolically Distinct Forms of Calf Thymus F3 Histone, Fed. Proc. 31, 3905, 1972.

Miller, D.M., Marzluff, W.F., Sanders, L.A., and McCarty, K.S. Specificity of Acetylation of Histones F3 and F2a1 from Normal and Leukemic Lymphocytes, Proc. Amer. Assn. Can Res. 13, 64, 1972.

Sanders, L.A., and McCarty, K.S., Isolation and Purification of Histones from Avian Erythrocytes, Biochemistry 11, 4216, 1972.

Marzluff, W.F., Miller, D.M., and McCarty, K.S., Occurrence of ε-N-Acetyllysine in Calf Thymus Histone F2b, Arch. Biochem. Biophys. 152, 472, 1972.

Supervisor of Ph.D. Thesis, Miller, D.M., Characterization of the Acetylation of the Arginine-Rich and Slightly Lysine-Rich Histones. (July) 1972.

Supervisor of Ph.D. Thesis, Miller, R.D., A Study of the Changes in Histones During Evolution and Development. (August) 1972.

1971

Hoerz, W., and McCarty, K.S., Initiation of Protein Synthesis in a Rabbit Reticulocyte Lysate System. Biochim. Biophys Acta 228, 526, 1971.

Sy, J., and McCarty, K.S., Formation in vitro of a 5.8S-26S Sea Urchin rRNA Complex. Biochim. Biophys. Acta 228, 517, 1971.

Supervisor of Ph.D. Thesis, Hoerz, W., Initiation of Protein Synthesis in a Rabbit Reticulocyte Lysate System. (Jan.) 1971.

Supervisor of Ph.D. Thesis, Marzluff, W.F., A Study of the Species Specificity of Histone Acetylation. (June) 1971.

McCarty, K.S., and Miller, D., Histone Acetylation in Heart Cells in Culture, Presented at Lake Placid, TCA Meeting June 1971.

McCarty, K.S., Chairman on Histones at Amer. Soc. Biol. Chemists, San Francisco June 1971.

McCarty, K.S., Protein Nucleic Acid Interactions in the Control of Hormone Induced Transcription in Mammary Tissue Cultures. Invited paper at Gordon Research Conference on Hormones, Aug. 1971.

McCarty, K.S., Transcriptional Controls in Mammalian Cells. Invited paper at Jackson Memorial Laboratories, Aug. 1971.

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1970

Sy, J., and McCarty, K.S., Characterization of 5.8S RNA from a Complex with 26S Ribosomal RNA from Arbacia Punctulata. Biochim. Biophys. Acta 199, 86, 1970.

Hoerz, W., and McCarty, K.S., In Vitro Ribosomal Subunit Associations in Protein Synthesis using Rabbit Reticulocyte Lysates. Fed. Proc. 29, 2000, 1970.

Marzluff, W.F., Miller, D., and McCarty, K.S., Histone Acetylation in Developing Mouse Mammary Gland in Vitro. Amer. Assoc. Cancer Res. 11, 52, 1970.

Marzluff, W.F. and McCarty, K.S., Two Classes of Histone Acetylation in Developing Mouse Mammary Gland. J. Biol. Chem. 245, 5635, 1970.

Wilhelm, J.A., and McCarty, K.S., Partial Characterization of the Histones and Histone Acetylation in Cell Cultures. Cancer Res. 30, 409, 1970.

Wilhelm, J.A., and McCarty, K.S., The Uptake and Turnover of Acetate in HeLa Cell Histone Fractions. Cancer Res. 30, 418, 1970.

Supervisor of Ph.D. Thesis, Sy, J., Characterization of Sea Urchin, Arbacia Punctulata, Ribonucleic Acids. (June) 1970.

1969

Hoerz, W., and McCarty, K.S., Evidence for a Proposed Initiation Complex for Protein Synthesis in Reticulocyte Polyribosome Profiles, Proc. Natl. Acad. Sci. 63, 1206, 1969.

Marzluff, W.F., McCarty, K.S., and Turkington, R.W., Insulin-Dependent Synthesis of Histones in Relation to the Mammary Epithelial Cell Cycle. Biochim. Biophys. Acta 190, 517, 1969.

Sy, J., and McCarty, K.S., Characterization of "7S" rRNA of Arbacia Punctulata, Fed. Proc. 28, 726, 1969.

Hijmans, J., and McCarty, K.S., Effect of Hydrocortisone on Chick Duodenum Cultured in Chemically Defined Medium. Proc. Soc. Exptl. Biol. Med. 131, 1407, 1969.

Supervisor of Ph.D. Thesis, Wilhelm, J., Partial Characterization of Histone Acetylation in HeLa Cells. (Mar) 1969.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)

	% time	Amount
Gerald M. Rosen	30	-------
Kenneth McCarty	20	--------

Technical

Technician	50	$4,000.00
	fringe benefit	$ 484.00

Sub-Total for A $4,484.00

B. Consumable supplies (by major categories)

isotopes,$1,000; animals,$1,000;
chemicals and glassware,$2,000

Sub-Total for B $4,000.00

C. Other expenses (itemize)

none

Sub-Total for C _____

Running Total of A + B + C $8,484.00

ok
MD

D. Permanent equipment (itemize)

balance,$1,000; pH meter,$750; rotary vacuum evaporator,$500; desk calculator,$150.

In the case of the balance, pH meter and desk calculator, I borrowed these from Dr. T. Narahashi, of this Dept. This arrangement can no longer be continued. The evaporator is needed in the synthesis of the compounds discussed.

Sub-Total for D $2400.00

E $1272.60

E. Indirect costs (15% of A+B+C)

Total request $12,156.60

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	4775	4000		2000	1316	12,091
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Development of Sydnone Nitrogen Mustards as Potential Antineoplastic Agents	N.I.H.	16,450	January 1, 1974- December 31, 1974

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Gerald M. Rosen

Signature Gerald M Rosen Date July 25, 1973

Telephone 919 (Area Code) 684-5549 (Number) (Extension)

Checks payable to

Duke Univerwsity

Responsible officer of institution

Typed Name W. G. Anlyan, M.D.

Title Vice President for Health Affairs

Mailing address for checks

C. B. Huestis
203 Allen Building
Duke University
Durham, North Carolina 27705

Signature W G Anlyan Date 7/27/73

Telephone 919-684-5175 (Area Code) (Number) (Extension)

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 21, 1973

Grant Application No. 709 CR1

TO: The committee comprising Drs. Andervont, Gardner and Huebner

SUBJECT: Lucio Severi, Professor, University of Perugia, Italy
Renewal application No. 709 CR1
"Identify agent (s) of sheep-lung adenomatosis and transfer to rodents"

History

This investigator has been supported by CTR since 1966, with successive re-orientations of his studies through the years.

Application 709 CR1 requests $19,948. for the second year of an approved three-year project. The initial estimate for this year was "approximately" $15,000.

The grant now in effect is for $15,000. a year.

Documents Submitted (attached)

1. Application dated 7th June, 1973.

2. Progress Report, 1st September, 1972 to 28th February, 1973.

The following three reprints are being sent to you separately:

Squartini, F. a. Bolis, G.B.: Extrachromosomic factors in spontaneous lung tumorigenesis of BALB/c/Cb/Se mice. Lav.Anat.Pat.Perugia 32, 5-16, 1973.

Biancifiori, C.: Cancerogensis in isotransplants of respiratory tissue from hydrazine treated BALB/c/Cb/Se mice. Lav.Anat.Perugia 32, 17-26, 1973.

Biancifiori, C.: Thyroid hyperplasias and pulmonary tumours induced by methylthiouracil and/or isoniazid in CBA/Cb/Se mice. Lav.Anat.Pat.Perugia 32, 27-39, 1973.

Comment

Attached is a memo from Dr. Huebner, dated June 8, 1973, on his site visit to Perugia during May 1973.

FWN:gh

F.W.N.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUN 19 1973

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐ Second Renewal ☐

Date: 7th June, 1973

1. Principal Investigator (give title and degrees):

Professor Lucio Severi, M.D.

2. Institution & address:

Division of Cancer Research,
University of Perugia,
P.O. Box 327,
06100 PERUGIA I T A L I A

3. Department(s) where research will be done or collaboration provided:

4. Short title of study:

Attempts to identify the viral agent(s) responsible for sheep-lung adenomatosis and to transfer this neoplastic disease to rodents

5. Proposed renewal date: 1st September, 1973

6. How results to date have changed earlier specific research aims:

No change

7. How results to date have changed earlier working hypothesis:

No change

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8. Any additional facilities now required? Describe briefly:

The Institute of Biochemistry will kindly supply facilities for the reverse transcriptase assays; but not the consumable supplies.

Fights to Sofia and Edinburgh for the collection of material ,owing to the difficulty in obtaining these in good condition.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

None

10. Append outline of experimental protocol for ensuing year. See pages 5 and 6 (attached)

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

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12. Summary progress report (append in standard form as separate document, unless recently submitted).

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Prof. Lucio Severi	5%	
Dr C. Biancifiori	33.3%	2,000
Dr R. Ribacchi	40%	3,500
Dr E. Bucciarelli	33.3%	1,800
Dr G.B. Bolis	33.3%	1,200
Technical Histological Technician		1,200
Sub-Total for A		$9,700

B. Consumable supplies (by major categories)	
Sheep samples and other material	1,000
Animal food and sawdust	600
Media, and chemical and biological supplies	1,000
Insturments and glassware	600
Staining materials	125
Sub-Total for B	3,325

C. Other expenses (itemize)	
Reverse transciptase assays (Consumable supplies for 30 tests)	1,300
Flight expenses for collection of fresh material	2,500
Sub-Total for C	3,800
Running Total of A + B + C	16,825

D. Permanent equipment (itemize)	
300 Animal cages	600
Sub-Total for D	600
E. Indirect costs (15% of A+B+C)	2,523
Total request	$19,948

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	NONE		

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mailing address for check:

Principal investigator

Typed Name Prof. Lucio Severi, M.D.

Signature Lucio Severi Date 14.VI.1973

Telephone 075 23356
Area Code Number Extension

Responsible officer of institution

Typed Name Prof. Lucio Severi, M.D.

Title

Signature Lucio Severi Date 14.VI.1973

Telephone 075 23356
Area Code Number Extension

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10. Outline of experimental protocol for ensuing year

As reported in the first Semi-annual Progress Report, sheep-lung adenomatosis cell cultures developed a cytopathic change characterized by small, round, fast-growing cells, which piled up, between the 20th and 35th days in culture. Normal fibroblast-like cells, derived from a lamb testicle, and a fibroblast-like cell line (AdP2a), derived from a sheep-lung adenomatosis, were maintained under the same culture conditions but did not show any cytopathic change.

The same cytopathic changes in cell cultures of sheep-lung adenomatosis from Kenya were described by Malmquist et al. (Lab. Invest 26, 528, 1972) and, in addition, they showed that the cytopathic change could be transmitted by cell-free tissue culture fluids of an adenomatosis case to a fetal sheep-lung cell. The transformed cells contained herpes like particles.

Thus the results we have reported do not change the earlier aims and they reinforce the earlier working hypothesis.

The experimental programme for the coming year will cover the following points:

1) The starting of cell culture lines of 8-10 cases of sheep lung adenomatosis. The sources of specimens are the same as those reported in the original research project, especially the Moredun Institute, Scotland.

   This will give us information regarding the diffusion and significance of the cytopathic changes we have described.

2) Search for sheep RNA C-type particles
   This research will be carried out on cells cultures of all adenomatosis cases and on fetal sheep fibroblasts, before and after iododeoxiuridine and/or bromodeoxiuridine. The C-type particle identification will be studied by:

   A. Reverse transcriptase tests on tissue culture fluids

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B. Immunofluorescence for gs-3 antigens with a very sensitive immunserum which will be supplied by Dr Robert J. Huebner.

C. Immunodiffusion for gs-3 antigens, on tissue culture fluid extracts, before and after ether treatment. I/D will also be performed on the original adenomatosis tumours extracts.

D. Electronmicroscopy of cell cultures and primary lung tumours in different conditions.

3) Search for herpes-like particles

A. Attempts to transfer cytopathic change to fetal sheep-lung cells by cell-free filtrates of tissue culture fluids and homogenate of primary tumours.

B. Intensive electron microscopy study of cells affected by cytopathic changes.

C. Attempts to transfer the disease to rodents.

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MEMORANDUM

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH

TO : CTR members Drs. Hockett, Kreisher, Sommers, Gardner

DATE: June 8, 1973

FROM : Dr. Robert Huebner RJH

SUBJECT: Site Visit to Dr. Severi's Laboratory at the Division of Cancer Research, University of Perugia, Perugia, Italy.

I drove a rental car from Rome to Perugia on May 28, 1973 for a meeting with Severi's group on May 29; I drove back to Rome on May 30. My expenses were $150.00.

In attendance at the meeting were Drs. Severi, Biancifiori, Bolis, Bucciarelli and Ribacchi.

The work on the CTR grant concerned with sheep lung adenomatosis (carcinoma?) is being carried on primarily by Ribacchi and Bucciarelli. This seemed quite appropriate since these two younger investigators are very much "with it" concerning recent work and literature concerning the role of RNA tumor viruses as likely causes of leukemia and cancer and they are particularly familiar with recent observations and publications by Scottish (Edinburgh) and Israeli workers which suggest that RNA tumor viruses are involved in the etiology of this lung cancer disease. The virus resembles type C and is distinct from visna virus, another RNA virus of sheep which is different in its pathogenic, biochemical and morphological properties.

During the period of the grant, Ribacchi and Bucciarelli succeeded in establishing and propagating for 22 subcultures a transformed "tumor" cell line from ovine adenomatosis lung tissue obtained from the University of Edinburgh (see pictures enclosed). Unfortunately, the line was lost at 23rd subculture. Extensive attempts to obtain fresh lung tumors from local abattoirs in Umbria have been fruitless. It appears now that the best sources available are Scotland and Sardinia. In order to get worthwhile specimens, Ribacchi feels that it is necessary for him to actually go and collect them personally. I agree with this assessment. His one successful cell culture was derived from the only tumor so far made available and all future efforts in his laboratory depend upon the feasibility of obtaining sufficient tumors from the field. This will require funds earmarked for this purpose which apparently are not currently available. I suggested therefore that they approach this supply problem forthrightly in their new proposal.

When I suggested that the original tumors and the cultures derived from them should have been tested regularly for the RNA dependent DNA polymerase as a sensitive indicator of virus, Ribacchi replied that they realized this

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but did not have the biochemical expertise available. They are, however, arranging now for such tests with the Department of Biochemistry at the University of Perugia. In reply to my question about the availability of the necessary equipment (collectors and counters) to do the test, Ribacchi stated that he was exploring this and would include such information in the proposal. I pointed out that at any point at which they succeeded in demonstrating RDP or type C viruses in tumor cell cultures, I could arrange through our NCI program to have such results confirmed and carried toward a more specific diagnosis.

In summary, Ribacchi and Bucciarelli have been working to establish in vitro cultures of ovine lung carcinoma. They have had a measure of success (see prints); they are knowledgeable concerning the state of the art and are eager to develop a laboratory test system for isolating the virus observed in lung tumors and cell cultures in Israel. From their work with the BALB/c mouse system, I feel they have a reasonably good prospect for doing this provided the tumor tissues are made available in good condition and in sufficient amounts to permit identification of viruses being observed by electron microscopy (Bucciarelli). Such materials would be useful for studies of the effects of the putative lung tumor virus in experimental animals.

Since there are no limitations against importation of such material into Italy where the disease is already endemic and since Ribacchi has made contacts in Edinburgh and plans to do likewise in Sardinia, the only limitations appear to be travel funds for acquisition of suitable materials. Materials sent by mail have proven worthless.

Perhaps it would be desirable for John Kreisher or some other Council Staff person to be assigned to help with acquisition of suitable supplies from areas known to have frequent lung adenomatosis. Unfortunately, nothing can be done about this problem in the U.S. since such materials would not be allowed to enter here. Inoculation of sheep lung tumor cells into lungs of BALB/c mice resulted in transfer in a number of unexpected tumors, but not lung tumors. I suggested that they study these tumors for virus which might be different from the mouse viruses.

Of the two or three groups now working on this problem, I believe that Ribacchi and Bucciarelli have as good a chance as any to establish the necessary cultures and to identify and culture the type C RNA virus already seen in the tumors by electron microscopy. Once isolated the virus could also then be worked with in other laboratories and compared with other slow sheep viruses such as visna. The availability of a specific lung cancer-inducing virus would prove to be very interesting not only to the Council but also the entire research community concerned with the etiology of cancer.

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I have promised to send Ribacchi diagnostic antiserum capable of detecting the interspecies specific (gs-3) antigen of the mammalian RNA tumor viruses. He will need this to identify the virus when present in lung tumors (tissues and cultures).* We will also supply specific antisera to gs-1 of the mouse tumor viruses so that Ribacchi, Bucciarelli and Biancifiori can wrap up their previous CTR-supported studies of type C virus in BALB/c lung tumors.

* S. Shiflett: Should this be goat antigen to FeLV virus? Call Gilden for best materials.

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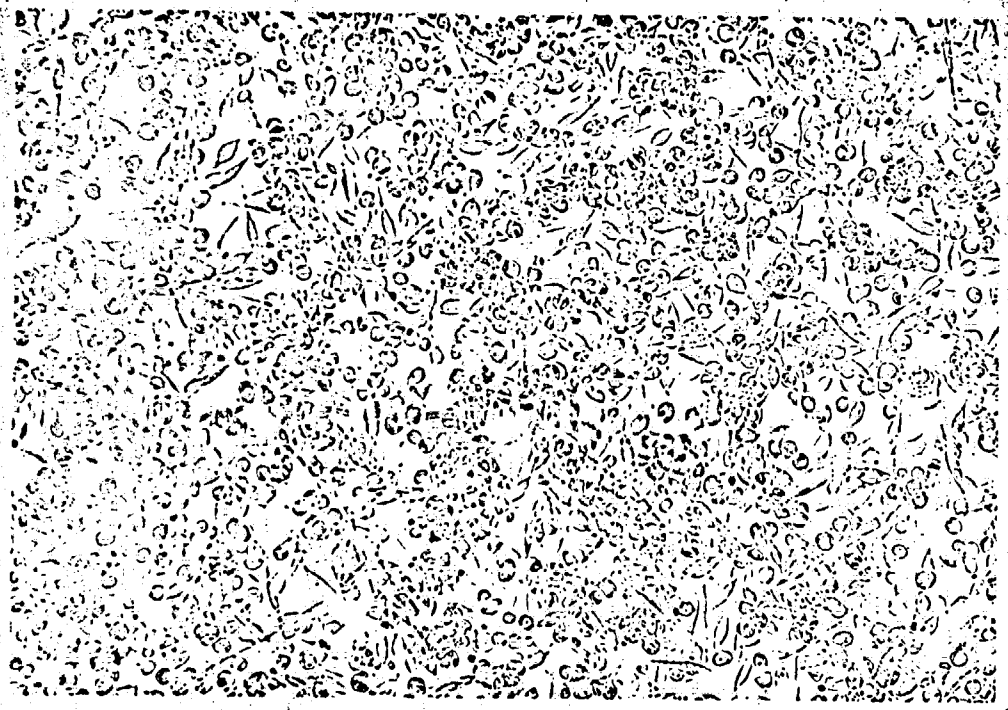

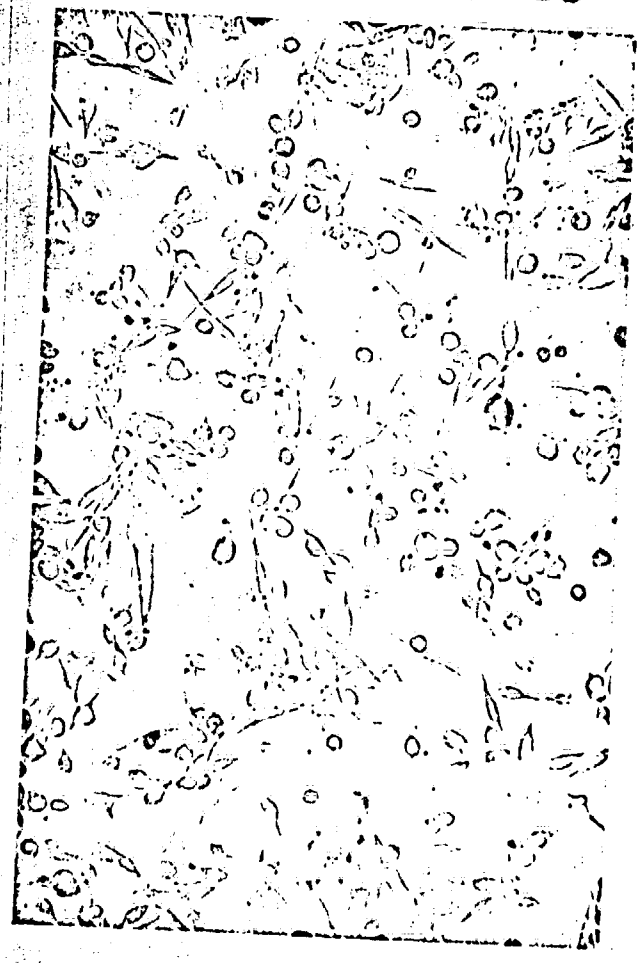

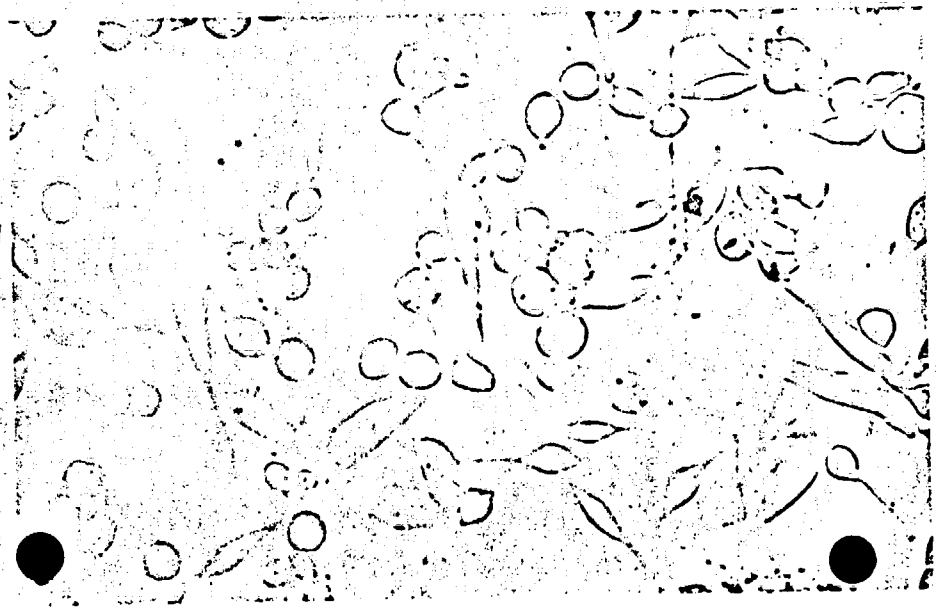

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#933 - SHAW

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

August 6, 1973

Grant Application No. 933
CANCER

To: The committee comprising Drs. Andervont, Gardner, Huebner, and Loosli

Subject: Charles R. Shaw, M.D., M. D. Anderson Hospital and Tumor Institute, Houston
New application No. 933
"Hydrocarbon Metabolizing Enzymes and Lung Cancer"

History

This proposal was Case No. 217 and application was encouraged.

Application No. 933 requests $76,721, plus two additional years.

Documents Submitted

Attached is application dated July 17, 1973 and copies of publications a) through e), page 11 of application.

F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: July 17, 1973

1. Principal Investigator (give title and degrees):

Charles R. Shaw, M.D.
Biologist; Chief, Section of Medical Genetics
and Professor of Biology

2. Institution & address:

The University of Texas at Houston
M. D. Anderson Hospital and Tumor Institute
6723 Bertner Avenue
Houston, Texas 77025

3. Department(s) where research will be done or collaboration provided:

Department of Biology, Section of Medical Genetics

4. Short title of study:

Hydrocarbon Metabolizing Enzymes and Lung Cancer

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: Three (3) years

7. Brief description of specific research aims:

a) To clarify the relationships between the variations in the human population of certain of the carcinogen-metabolizing enzymes and the occurrence of lung cancer.

b) To modify the method, developed in our laboratory, for assay of aryl hydrocarbon hydroxylase in human subjects, for adaptation as a clinical laboratory procedure.

c) To compare activities of these carcinogen-metabolizing enzymes in lung, cultured lymphocytes, and other tissues.

d) To investigate the effects of inherently different levels of these enzymes on rates of incorporation of carcinogen metabolites into cell components and the effects of these metabolites on cell activities, particularly DNA repair mechanisms and *in vitro* transformation.

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8. Brief statement of working hypothesis:

Most of the hydrocarbon carcinogens are converted in the target cell to the reactive epoxide form of the molecule, by aryl hydrocarbon hydroxylases (AHH). This is an inducible enzyme, and the degree of inducibility is genetically variable in man. We hypothesize that the level of AHH determines, at least in part, susceptibility to chemical carcinogenesis. Our preliminary studies in man support this hypothesis.

9. Details of experimental design and procedures (append extra pages as necessary)

The basic design of the clinical research is to measure AHH activity and inducibility in lung cancer subjects and their families, employing the lymphocyte culture system developed in our laboratory (Busbee *et al.*, 1972). This assay has been shown to be a competent indicaton of the general, hereditarily-determined inducibility of this enzyme in the individual. In the normal population there are three distinct groups, having low, intermediate, and high levels of inducibility (Kellermann *et al.*, 1973a). Preliminary studies of fifty lung cancer cases indicate that only persons with intermediate and high levels are susceptible to this disease. Larger and more detailed studies are indicated, to determine if those with highest AHH activities are more susceptible than intermediates (earlier onset, greater severity of disease, etc.), and which types of bronchogenic carcinoma are effected through the AHH system. It appears that the oat cell carcinoma is in a different category. Family studies of the lung cancer subjects are indicated, to clarify cause-effect relationships. Such studies will show whether the subjects fall into the expected hereditary groups, or whether their increased inducibility could be a result of the cancer. The latter is unlikely, but needs to be examined.

Lung tissue, both normal and diseased, will also be studied, by organ and tissue culture, to correlate enzyme activities with that in cultured lymphocytes. Other enzymes will also be studied in these tissues, particularly those which effect the breakdown of the epoxides and especially the epoxide hydrase. Preliminary studies in our laboratory suggest that the epoxide hydrase is not a rate-limiting enzyme in hydrocarbon metabolism (Kellermann *et al.*, 1973b).

To investigate effects of varying levels of AHH activity in the cell, cell cultures will be established from subjects with low and high levels. Rate of incorporation of labeled hydrocarbon metabolites into these cultured cell lines will be determined, and if possible, localization of binding sites will be identified. Further, effects of incorporation of these metabolites on DNA repair mechanisms will be measured in collaboration with Dr. Roger Hewitt of this depantment. These studies will be directed mainly to previously characterized DNA repair enzymes.

Efforts will be made to develop the AHH assay system as standardized clinical laboratory procedure. This has a number of obvious applications: as a diagnostic tool, in mass screening procedures to determine susceptibility to lung cancer and possibly other chemically induced cancers, and to study gene frequencies among various human populations. Our studies indicate that approximately half of the people in the U.S. population having low AHH inducibility, carry an extremely low risk for lung cancer due to smoking. The higher-risk individuals may be identified and counselled appropriately.

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratories of the principal investigator are located on the fifth floor of the Research Institute Wing of The University of Texas M. D. Anderson Hospital and Tumor Institute. They consist of five laboratories totaling 1,600 square feet, with a walk-in cold room and four adjoining offices. They are well equipped for general biochemistry, and major items include a Nuclear-Chicago Unilux IIA Scintillation Counter, two Gilford recording spectrophotometers (Models 2400 and 240), Beckman L-4 preparative ultracentrifuge, 12 complete sets of starch gel electrophoresis apparatus, two Sorvall RC-2 B refrigerated high speed centrifuges, an incubator, and a large amount of column chromatography apparatus.

Additionally available for our use located in adjoining areas of the Department of Biology are a glassware preparations room, a walk-in incubator, and a variety of other biochemical instruments available for part-time use. This includes an Aminco-Bowman spectrophotofluorometer, which is the main instrument employed in the present investigation.

The clinical facilities of the M. D. Anderson Hospital and Tumor Institute are available for this study. The M. D. Anderson Hospital and Tumor Institute is a categorical institution for the study and treatment of neoplastic diseases. It has approximately 300 inpatient beds, and an addition is presently under construction which will add another 300. Patients are seen by referral only and the outpatient clinic sees approximately 6,000 referrals annually.

11. Additional facilities required:

Our pressing need for this project is for several items of equipment. A spectrophotofluorometer is, as noted above, the instrument employed for measuring the hydroxylase activity. We are presently using an instrument in the laboratory of Dr. Roger Hewitt, and his instrument is heavily used and at times not available to us. A CO_2 incubator is needed for the organ and cell culture work. For preparation of lymphocytes and cell cultures a refrigerated centrifuge is required. A phase-optics microscope is needed to evaluate the condition of cultured cells and to count the cells before and after culture.

Otherwise, the present facilities and equipment are quite adequate for this project.

12. Biographical sketches of investigator(s) and other professional personnel (append):

Charles R. Shaw, M.D., Elroy Cantrell, Ph.D., and Gottfried Kellermann, Ph.D. (Please see attached)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Attached

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R: REDACTED MATERIAL

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Charles Raymond Shaw	Biologist and Professor of Biology; Chief, Section of Medical Genetics	REDACTED
PLACE OF REDACTED (City and country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) R	SEX ☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Hanover College, Hanover, Indiana	None		Pre Med
University of Alabama, Tuscaloosa, Alabama	None		Medicine
New York University School of Medicine, New York, New York	M.D.	1946	Medicine

HONORS

Gamma Sigma Pi
Diplomate, American Board of Psychiatry and Neurology
Sigma Xi

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Mammalian Biochemical Genetics	Principal Investigator

RESEARCH SUPPORT *(See instructions)* PENDING GRANTS:

NIH Research Grant #5-R01-GM-15597, Genetic and Comparative Studies of Enzymes
09-01-68 through 08-31-73, $267,000 (direct costs)
Support during current year 09-01-72 through 08-31-73, $58,000 (direct costs)
(up for competitive renewal)

NIH Research Grant #1 R01 CA 14196, Enzymatic Modification of Chemical Carcinogens
01-01-73 through 12-31-77, $282,018 (direct costs)
Support during first year, 01-01-73 through 12-31-74, $57,426 (direct costs)
(approved but not funded, being held in active file until 31 December 1973)

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)*

Chief, Section of Medical Genetics, Department of Biology, The University of Texas at Houston M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1968 - present.
Biologist and Professor of Biology, Department of Biology, The University of Texas at Houston M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1969 - present.
Member, Graduate Faculty, The University of Texas at Houston Graduate School of Biomedical Sciences, Houston, Texas, 1969 - present.
Member, Senior Faculty, Medical Genetics Center, The University of Texas at Houston Graduate School of Biomedical Sciences, Houston, Texas, 1972 - present.
Associate Biologist and Associate Professor of Biology, The University of Texas at Houston M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1967-69.
Director, Biological Research Unit, Hawthorn Center (children's psychiatric hospital), Northville, Michigan, 1958-67.
Senior Staff Child Psychiatrist, Hawthorn Center, Northville, Michigan, 1956-58.
Resident in Psychiatry, Neuropsychiatric Institute, University of Michigan Medical School Ann Arbor, Michigan, 1953-56.
Chief, Nutrition and Metabolism Section, Arctic Aeromedical Laboratory, Fairbanks, Alaska, 1951-53.
Assistant Professor of Nutrition, Cornell University School of Nutrition, Ithaca, New York, 1949-51.
Resident, Internal Medicine, Cornell Infirmary and Memorial Hospital, Ithaca, New York, 1947-49.
Member: Am. Soc. Human Genet., Am. Psychiatric Assoc., Environmental Mutagen Soc., Genet. Soc. Am., Am. Soc. Zool., Aircraft Owners and Pilots Assoc.

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PUBLICATIONS - Dr. Charles R. Shaw

Shaw, C. R. and E. Barto: Genetic Evidence for the Subunit Structure of Lactate Dehydrogenase Isozymes. Proc. Nat. Acad. Sci. U.S. 50: 211, 1963.

Koen, A. L. and C. R. Shaw: Multiple Substrate Specificities of Some Dehydrogenase Molecules. Biochem. Biophys. Res. Comm. 15: 92, 1964.

Shaw, C. R.: The Use of Genetic Variants in the Analysis of Isozyme Structure. Proc Brookhaven Symposia in Biology 17: 117, 1964.

Koen, A. L. and C. R. Shaw: A Preparative Method Employing Starch Gel Electrophoresis and Electrodialysis. Analyt. Biochem. 9: 495, 1964.

Shaw, C. R. and A. L. Koen: Aspartate Dehydrogenase Activity of Malate Dehydrogenase Biochim. Biophys. Acta 92: 397, 1964.

Shaw, C. R. and E. Barto: Autosomally Determined Polymorphism of Glucose-6-Phosphate Dehydrogenase in Peromyscus. Science 148: 1099, 1965.

Shaw, C. R.: Electrophoretic Variation in Enzymes. (lead article) Science 149: 936, 1965.

Shaw, C. R.: Glucose-6-Phosphate Dehydrogenase: Homologous Molecules in Deer Mouse and Man. Science 153: 1013, 1966.

Shaw, C. R. and A. L. Koen: Galactose Dehydrogenase, "Nothing" Dehydrogenase and Alcohol Dehydrogenase: Interrelation. Science 156: 3781, 1967.

Shaw, C. R. and A. L. Koen: Tissue-Specific Variation in Glucose-6-Phosphate Dehydrogenase Isozymes of Man and Deer Mouse. Ann. N. Y. Acad. Sci. 151: 149, 1968.

Shaw, C. R.: Isozymes: Classification, Frequency and Significance. Int'l. Rev. Cytol. 25: 297, 1969.

Shaw, C. R.: The Molecular Basis of Isozymes. Jap. J. Genet. 44, Suppl. 1: 31, 1969.

Wright, D. and C. R. Shaw: Genetics and Ontogeny of α-Glycerophosphate Dehydrogenase Isozymes of Drosophila melanogaster. Biochem. Genet. 3: 343, 1969.

Baptist, J., C. R. Shaw, and M. Mandel: Zone Electrophoresis of Enzymes in Bacterial Taxonomy. J. Bacteriol. 99: 180, 1969.

Shaw, C. R.: How Many Genes Evolve? Biochem. Genet. 4: 275, 1970.

Shaw, C. R. and R. Prasad: Starch Gel Electrophoresis of Enzymes--A Compilation of Recipes. Biochem. Genet. 4: 297, 1970.

Wright, D. A. and C. R. Shaw: Time of Expression of Genes Controlling Specific Enzymes in Drosophila Embryos. Biochem. Genet. 4: 385, 1970.

Busbee, D. L., C. R. Shaw, and E. T. Cantrell: Aryl Hydrocarbon Hydroxylase Induction in Human Leukocytes. Science 178: 315, 1972.

Nevo, Eviatar and Charles R. Shaw: Genetic Variation in a Subterranean Mammal, Spalax ehrenbergi. Biochem. Genet. 7: 235, 1972

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Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

Shaw, C. R., M. J. Siciliano, and D. A. Wright: Inter- and Intra-specific Genetic Distances Among Teleosts. Proc. Int'l. Cong. Zool., 1972, in press.

Stout, Daniel L. and Charles R. Shaw: Comparative Enzyme Patterns in Two Species of Thamnidium. Mycologia, in press.

Shaw, C. R.: Human Biochemical Variation. In Human Behavior Genetics, ed. A. R. Kaplan, M.D., in press.

Shaw, C. R. and R. Prasad: Genetic Variants of Enzymes Detectable by Zone Electrophoresis. In Handbook of Genetics, ed. Dr. R. C. King, Van Nostrand Reinhold Co., New York, in press.

Shaw, C. R., J. N. Baptist, D. A. Wright, and T. S. Matney: Induction of a Mutation in E. coli Affecting the Electrophoretic Mobility of Enzymes. Mutation Research 18: 247, 1973.

Kellermann, G., E. Cantrell, and C. Shaw: Variation in Inducibility of Aryl Hydrocarbon Hydroxylase in Human Leukocytes. Cancer Research, in press.

Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Genetic Variation of Aryl hydrocarbon hydroxylase in Human Lymphocytes. American Journal of Human Genetics 25: 327, 1973.

Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Presence and Induction of Epoxide Hydrase in Cultured Human Leukocytes. Biochemical and Biophysical Research Communications 52: 712, 1973.

Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Metabolism of Polycyclic Aromatic Hydrocarbons in Cultured Human Leukocytes under Genetic Control. Submitted to Humangenetik.

Kellermann, G., C. R. Shaw, and M. Luyten-Kellermann: Aryl Hydrocarbon Hydroxylase Inducibility and Bronchogenic Carcinoma. Submitted to The New England Journal of Medicine.

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BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Elroy Taylor Cantrell	Research Associate	REDACTED

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
REDACTED	REDACTED	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Arkansas State University, Jonesboro, Ark	B.S.	1965	Biology (chemistry)
University of Tennessee Medical Units, Memphis, Tennessee	M.S.	1968	Pharmacology
Baylor College of Medicine, Houston, Texas	Ph.D.	1972	Pharmacology(cell biolo
Baylor College of Medicine, Houston, Texas			Postdoctoral Fellow

HONORS

Phi Eta Sigma - Arkansas State University
Honors System - Arkansas State University
Beta Beta Beta - Arkansas State Univeristy

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Metabolism of carcinogens	Research Associate

RESEARCH SUPPORT *(See instructions)*

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)*

Research Associate, The University of Texas Graduate School of Biomedical Sciences Medical Genetics Center, Houston, Texas, August, 1972 to present.

Postdoctoral Fellow. Studies of effect of Flavin deficiency on drug metabolism, studies of benzpyrene metabolism in human leukocytes and human pulmonary alveolar macrophages. Studies of effects of antimetabolites on components of mixed-function oxidases and enzyme activities. October 1971 - July, 1972.

Graduate student. Isolation of cell types in liver to study contribution of each in drug metabolism. Histofluorescence analysis for drug metabolism. Analysis of cytochromes b_5 and P-450 in hepatocytes and Kupffer cells. Some experience in electron microscopy, mass spectrometry, tissue culture, disc gel electrophoresis, and animal surgery. September, 1968 - October, 1971.

Graduate student. Studies of functional dynamics of the reticuloendothelial system and its relationship to drug metabolism. September, 1965 - September, 1968.

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PUBLICATIONS - Dr. Elroy Cantrell

Cantrell, Elroy T.: Induction of benzpyrene hydroxylase in parenchymal and Kupffer cells of rat liver. Ph.D. Dissertation, Department of Pharmacology, Baylor College of Medicine, Houston, Texas. 1971.

Cantrell, E., and Bresnick, E.: Evidence for type II enzyme induction by β-naptho-flavone. Life Sci. 10: 1195, 1971.

Cantrell, E. and Bresnick, E.: Benzpyrene hydroxylase activity in parenchymal and non-parenchymal cells of rat liver. J. Cell. Biol. 52: 316, 1972.

Cantrell, E. T., Martin, R. R., Warr, G. A., Busbee, D. L., Kellermann, G., and Shaw, C. R.: Induction of aryl hydrocarbon hydroxylase in human pulmonary alveolar macrophages by cigarette smoking. Submitted for publication, 1972.

Cantrell, E. T., Busbee, D. L., Kellermann, G., and Shaw, C. R.: Effects of mitogens and 3-methylcholanthrene on aryl hydrocarbons hydroxylase in cultured human lymphocytes. In preparation, 1972.

Black, O., Cantrell, E., Buccino, R. J., and Bresnick, E.: Effects of 3-methylcholanthrene administration on the proteins of endoplasmic reticulum. Biochem. Pharmacol. 20: 2989, 1971.

Busbee, D. L., Shaw, C. R., and Cantrell, E. T.: Aryl hydrocarbon hydroxylase induction in human leukocytes. Science 178: 315, 1972.

Kellermann, G., Cantrell, E., and Shaw, C. R.: Variation in inducibility of aryl hydrocarbon hydroxylase in human leukocytes. Submitted to Cancer Research.

Abstracts:

Cantrell, E. R., Cantrell, W. F., and Elko, E. E.: Sulfadiazine acetylation and phagocytic activity during liver regeneration in the rat. Pharmacologist 10: 191, 1968.

Cantrell, E. T., Burki, K., and Bresnick, E.: In vitro elevation of benzpyrene hydroxylase activity by 3-methylcholanthrene in rat hepatocytes. Southwest Section American Association for Cancer Research, October 16-17, 1970.

Cantrell, E.: Benzpyrene hydroxylase induction in parenchymal and non-parenchymal cells of rat liver. Fed. Proc. 30: 506, 1971.

Cantrell, E. and Busbee, D.: Benzpyrene hydroxylase in circulating leukocytes after exposure to polycyclic hydrocarbons. Fifth International Congress on Pharmacology. San Francisco, July 23-28, 1972.

Cantrell, E., Martin, R., Warr, G., and Shaw, C. R.: Aryl hydrocarbon hydroxylase induction in human alveolar macrophages. Clirical Research XX(4): 708, 1972.

Gerber, N., Seibert, R., Desiderio, D., Cantrell, E., and Lane, M.: Methodology for identification and quantification of 3,5-diamino-1,2,4-triazole, Guanazole (G), a new anticancer agent, and metabolic studies in man and animals. Fed. Proc. 31: 535 abs, 1972.

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BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Gottfried H. Kellermann	Postdoctoral Fellow in Biology	REDACTED
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If U.S. citizen, indicate kind of visa and expiration date)	SEX
REDACTED	REDACTED	☒ Male ☐ Female

EDUCATION (Begin with Baccalaureate or other initial professional education, such as Postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Philosophische Hochschule, Munich, Germany	M.S.	1967	Philosophy
University Frankfort, Mainz, Germany	M.S.	1968	Biology
University of Mainz, Germany	Ph.D.	1971	Human Genetics
University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute	Postdoctoral Fellow	1972 to present	Biochemical Genetic

HONORS

Cum Laude, Philosophische Hochschule, Munich, Germany
Magna Cum Laude, University of Mainz, Faculty of Natural Sciences.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Biochemical Genetics	Research Associate

RESEARCH SUPPORT *(See instructions)*

Fellowship, Deutsche Forschungsgemeinschaft (DFG) Grant KE 217, May 1972 through June, 1974.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)*

Postdoctoral Fellow, The University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1972 to present.

Assistant Biologist, Anthropologisches Institute der Johannes Guternberg-Universitat, Mainz, Germany, 1971 to 1972.

Kellermann, G. and H. Walter: Investigation on the Population Genetics of the α_1-Antiserum Polymorphisms. Humangenetik 10: 145-150, 1970.

Kellermann, G. and H. Walter: On the Genetics of the Pi-Serum Proteins. Humangenetik 10: 191-194, 1970.

Walter, H., M. Bajatzadeh, G. Kellermann, and T. Matznetter: Associations Between Leprosy and Serum Protein Groups. Humangenetik 10: 298-308, 1970.

Kellermann, G.: Methodological Investigations on the ABO-Typing of Ancient Bones. Humangenetik 14: 50-55, 1971.

Kellermann, G.: Further Studies on the ABO-Typing of Ancient Bones. Humangenetik 14: 232-236, 1972.

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PUBLICATIONS - Dr. Gottfried Kellermann

Walter, H., G. Kellermann, M. Bajatzadeh, J. Krüger, and M. R. Chakravartti: Hp, Gc, Cp, Tj, Bg, and Pi-Phenotypes in Leprosy Patients and Healthy Controls from West Bengal (India). Humangenetik 14: 314-325, 1972.

Kellermann, G. and H. Walter: On the Population Genetics of the Ceruloplasmin Polymorphism. Humangenetik 15: 84-86, 1972.

Kellermann, G., E. Kleinman, and H. Walter: Zur Anwendbarkeit des Pi-Systems in der Vaterschaftskegutachtung Z. für Rechtsmedizin 71: 24-26, 1972.

Kellermann, G.: Paläoserologische Untersuchungen an Skelett funden aus dem 12. und 14. Jahrhundert. In press, 1973.

Kellermann, G., E. Cantrell, and C. Shaw: Variation in Inducibility of Aryl Hydrocarbon Hydroxylase in Human Leukocytes. Cancer Research, in press.

Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Genetic Variation of Aryl Hydrocarbon Hydroxylase in Human Lymphocytes. American Journal of Human Genetics 25: 327, 1973.

Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Presence and Induction of Epoxide Hydrase in Cultured Human Leukocytes. Biochemical and Biophysical Research Communications 52: 712, 1973.

Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Metabolism of Polycyclic Aromatic Hydrocarbons in Cultured Human Leukocytes Under Genetic Control. Humangenetik, in press.

Kellermann, G., C. R. Shaw, and M. Luyten-Kellermann: Aryl Hydrocarbon Hydroxylase Inducibility and Bronchogenic Carcinoma. Submitted to The New England Journal of Medicine.

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13. Publications

a) Busbee, D. L., C. R. Shaw, and E. T. Cantrell: Aryl Hydrocarbon Hydroxylase Induction in Human Leukocytes. *Science 178:* 315-316, 1972.

b) Cantrell, E. T., R. R. Martin, G. A. Warr, D. L. Busbee, G. Kellermann, and C. Shaw: Induction of Aryl Hydrocarbon Hydroxylase in Human Pulmonary Alveolar Macrophages by Cigarette Smoking. *Transactions of the Association of American Physicians.* In press.

c) Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Genetic Variation of Aryl Hydrocarbon Hydroxylase in Human Lymphocytes. *American Journal of Human Genetics 25:* 327-331, 1973.

d) Kellermann, G., M. Luyten-Kellermann, and C. R. Shaw: Presence and Induction of Epoxide Hydrase in Cultured Human Leukocytes. *Biochemical and Biophysical Research Communications 52:* 712-716, 1973.

e) Kellermann, G., C. R. Shaw, and M. Luyten-Kellermann: Aryl Hydrocarbon Hydroxylase Inducibility and Bronchogenic Carcinoma. Submitted to *The New England Journal of Medicine.*

(Reprints enclosed for publications a, c, and d. Preprints enclosed for publications b and e.)

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Charles R. Shaw, M.D.		REDACTED
Elroy Cantrell, Ph.D.		
Gottfried Kellermann, Ph.D.		
Technical		
To be recruited Research Technician III		
To be recruited Research Technician II		REDACTED
Fringe Benefits		1,529
Sub-Total for A		$ 20,301
B. Consumable supplies (by major categories)		
Chemicals		$ 8,000
Glassware		2,800
Culture media		8,000
Disposables		5,000
Sub-Total for B		$ 23,800
C. Other expenses (itemize)		
Volunteer human subjects:		$ 5,200
$10 x 400, blood specimens $ 4,000		
$35 x 20, pulmonary lavage 700		
$25 x 20, skin biopsy 500		
Travel, mostly local, to collect specimens from family members - private car and airplane		800
Sub-Total for C		$ 6,000
Running Total of A + B + C		$ 50,101
D. Permanent equipment (itemize)		
Spectrophotofluorometer, Aminco-Bowman, with ellipsoidel condensing system, XY recorder, extra cells and thin-film scanner		$ 12,695
Centrifuge, Beckman PR-J, with swinging bucket rotor		2,730
Incubator, CO_2, New Brunswick Model CO-20		1,350
Microscope, Nikon, with phase optics and 35 mm. camera		2,330
Sub-Total for D		$ 19,105
E. Indirect costs (15% of A+B+C)		7,515
Total request		$ 76,721

15. Estimated future requirements:
See attached

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$ 21,311	24,500	6,500	-0	7,847	$ 60,158
Year 3	$ 22,288	25,000	7,000	-0	8,143	$ 62,431

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15. Estimated future requirements:

Personnel

Salaries are budgeted in accordance with the Institution's Personnel Pay Plan. Fringe Benefits include employer contributions to Social Security, Workmen's Compensation Insurance, Unemployment Compensation Insurance, and employee's insurance program. These costs are all uniformly charged as direct costs to all grants and contracts.

Increases requested for personnel in subsequent years have been computed in accordance with the established institutional policy, and include a one-step increase for all employees in all additional years of the project.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Genetic and Comparative Studies of Enzymes (up for competitive renewal)	National Institutes of Health #2 ROI GM 15597-07	346,840	09-01-73 through 08-31-78

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Charles R. Shaw, M.D.

Signature Charles R. Shaw Date 17 July 1973

Telephone 713 (Area Code) 526-5411 (Number) 522 (Extension)

Checks payable to:
Mr. E. R. Gilley, Business Manager

Mailing address for checks:
The University of Texas at Houston
M. D. Anderson Hospital and Tumor Institute
6723 Bertner Avenue
Houston, Texas 77025

Responsible officer of institution:

Typed Name Robert C. Hickey, M.D.

Title Director

Signature Robert C. Hickey Date 7/27/73

Telephone 713 526-5411 544

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Publication a

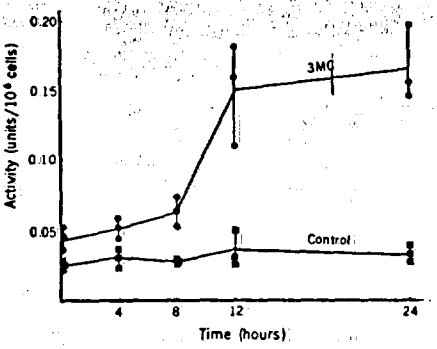
Aryl Hydrocarbon Hydroxylase Induction in Human Leukocytes

Abstract. *A method for determining aryl hydrocarbon hydroxylase induction in human leukocytes is described. Leukocytes from healthy volunteers were cultured in the presence of phytohemagglutinin, a mitogen. Addition of 3-methylcholanthrene to 72-hour cultures induced a fourfold increase in aryl hydroxylase activity. In the absence of a mitogenic agent, 3-methylcholanthrene stimulation of increased enzymatic activity did not occur.*

Benzo[*a*]pyrene (BP), 3-methylcholanthrene (3MC), and related polycyclic hydrocarbons are carcinogens commonly present in tobacco smoke (*1*), polluted city air (*2*), and certain foods (*3*). The enzyme system aryl hydrocarbon hydroxylase (AHH) functions in the biotransformation of BP and related compounds to hydroxylated metabolites with resultant alteration of their carcinogenic activity. The exact relation between AHH activity and resistance to tumor initiation by environmental carcinogens is not well understood. This inducible enzyme occurs in many mammalian tissues including liver, lung, intestinal mucosa, thyroid, testis, adrenal cortex (*4*), striated muscle (*5*), placenta (*6*), and skin (*7*, *8*). The inducibility of AHH shows genetic variation in the mouse, and is controlled by a single autosomal dominant locus (*9*). If this genetic control is as simple in man the implications would be profound.

Increased activity of AHH has been found in human placenta from cigarette smokers (*6*), and in cultured human foreskin from newborn infants (*8*). The lack of a method for assaying readily available human tissues has confined previous studies of AHH induction and activity to select populations. The demonstration of AHH induction in rat Kupffer cells (*10*) suggested agranular leukocytes (precursors of Kupffer cells) (*11*), may be inducible under appropriate conditions. We describe here a method for measuring the induction of AHH activity in normal human leukocytes.

To 10 ml of heparinized blood we added an equal volume of 3 percent dextran in normal saline and allowed the erythrocytes to sediment for 45 minutes at room temperature. The leukocyte-rich supernatant was centrifuged at 1000*g* for 3 minutes. The pellet was suspended in Gibco chromosome medium 1-A which contains phytohemagglutinin. Culture tubes were prepared containing 2×10^6 to 4×10^6 cells in 5 ml of medium. After incubation for specified intervals at 37°C appropriate tubes received 5 μl of 0.75 m*M* 3MC in methanol. Control tubes received only methanol. The cells were harvested 24 hours after addition of 3MC, and were suspended in 1.0 ml of 50 m*M* tris-HCl, *p*H 7.5, containing 3 m*M* $MgCl_2$ and 0.2*M* sucrose (TMS). Cell counts were made from the 1.0 ml cell suspension after a 1 : 1 dilution of a 25-μl portion with 1 percent acetic acid containing a trace of gentian violet. Leukocytes were homogenized in a glass tissue grinder, and



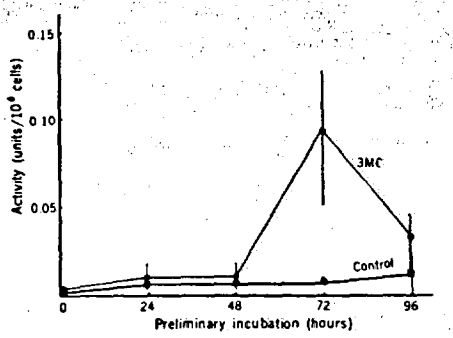


Fig. 1 (left). Time course of AHH induction by 3-methylcholanthrene (*3MC*) Leukocytes were first incubated for 72 hours in medium containing phytohemagglutinin. 3-Methylcholanthrene was added to a final concentration of 0.75 μM. Cells were harvested at the indicated intervals and were assayed for AHH activity. Each point represents a single determination; vertical bars represent the range of values. A unit of activity is the fluorescence produced equivalent to a picomole of 3-hydroxybenzopyrene per minute. Fig. 2 (right). Response of leukocytes to 3-methylcholanthrene during cultures. Leukocytes were maintained in phytohemagglutinin medium for the indicated times prior to addition of 3-methylcholanthrene and harvested after 24 hours. Each circle represents the mean of six determinations and each square represents the mean of three determinations. The vertical bars give the range of values.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 1, 1973

Grant Application No. 930
CANCER

To: The committee comprising Drs. Andervont, Huebner, and Jacobson

Subject: Andrew Sivak, Ph.D., N.Y.U. Medical Center
New application No. 930
"Chemical and Viral Factors in the Etiology of Neoplastic Transformation"

History

This proposal was Case No. 178, and formal application was encouraged. Application No. 930 requests $54,838 plus two additional years.

Documents Submitted

Attached is application dated July 25, 1973.

Copies of the papers listed on page 3c were provided, and will be forwarded to you if you wish.

F.W.N.

FWN:wg
Encl.

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CANCER

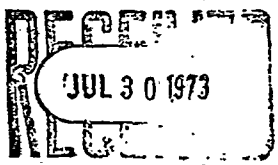
Comm.
Dr. Andervont
Dr. Huebner
Dr. Jacobson

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8385

Application for Research Grant
(Use extra pages as needed)

Date: July 25,1973



1. Principal Investigator (give title and degrees):

Andrew Sivak, Ph.D.
Associate Professor of Environmental Medicine

2. Institution & address:

New York University Medical Center
550 First Avenue
New York, New York 10016

3. Department(s) where research will be done or collaboration provided:

Environmental Medicine

4. Short title of study:

Chemical and Viral Factors in the Etiology of Neoplastic Transformation

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

7.a. To examine in mammalian cell culture systems the possible cooperative and synergistic effects of sequential exposure to two different classes of carcinogens by:

7.a.1. Measuring the effects of prior treatment of mammalian cells with chemical carcinogens, cocarcinogens or mutagens on subsequent neoplastic transformation by representatives of the 3 major known classes of oncogenic viruses.

7.a.2. Determining whether mammalian cells infected with these oncogenic viruses show enhanced expression of neoplastic transformation after treatment with chemical carcinogens, cocarcinogens or mutagens.

7.b. To determine whether the effects of the chemicals are due to alterations of the host cell DNA.

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8. Brief statement of working hypothesis:

Evidence from a variety of sources suggests that cells bearing certain types of genetic lesions are more susceptible than normal cells to additional DNA damage induced by chemicals or viruses, often resulting neoplastic transformation (G.J. Todaro, H. Green and M.R. Swift, Science, 153: 1252, 1966; S.A. Aaronson and G.J. Todaro, Virology, 36: 254, 1968; R.L. O'Brian et al., Intern. J. Cancer, 8: 202, 1971).

Thus, the exposure of cells in culture to carcinogenic chemicals and oncogenic viruses should provide an example for study of the increased carcinogenic risk from multiple exposures to different classes of etiologic agents that are potentially present in the environment. The prior treatment of cell populations with chemical carcinogens or mutagens is expected to result in a marked enhancement of the frequency of neoplastic transformation by subsequent exposure to oncogenic viruses. Similarly, the reversal of the sequence, i.e. the treatment of cells already infected by oncogenic viruses with appropriate chemical agents, especially cocarcinogens, should also yield increased transformation frequencies. Finally, some of these effects can be associated with demonstrable increases in damage to the host cell DNA.

9. Details of experimental design and procedures (append extra pages as necessary)

9.a. Chemicals - Carcinogens of several chemical classes will be used. All agents will be recrystallized or redistilled to ensure their purity. Compounds to be used will be selected from the following list:

9.a.1. Carcinogens (demonstrated as mutagens in various organisms)
7,12-Dimethylbenz(a)anthracene; benzo(a)pyrene; diepoxybutane; bis(chloromethylether); N-acetoxy-2-fluorenyl acetamide; N-methyl-N'-nitro-N-nitrosoguanidine; 4-nitroquinoline-N-oxide; aflatoxin B_1.

9.a.2. Cocarcinogens
Phorbol myristate acetate; anthralin; cigarette smoke condensate.

9.a.3. Mutagens not known to be carcinogens
Ethylmethane sulfonate; nitrogen mustard; ethidium bromide;

9.a.4. Noncarcinogens
Benzo(e)pyrene; 1,2,3,4-diepoxycyclohexane

9.b. Biological Systems

9.b.1. BALB/c-3T3 Cells + SV_{40} Virus

This transformation assay will be carried out essentially as described by Todaro and Green (Proc. Nat. Acad. Sci., 55: 302, 1966; Virology, 28: 756, 1966) and adapted for BALB/c-3T3 cells (S.A. Aaronson and G.J. Todaro, J. Cell. Physiol., 72: 141, 1968). Logarithmically growing cell populations will be exposed to the chemical agents to be tested for intervals up to 48 hours. The concentrations of the chemicals will be selected so that the highest doses result in no more than a 90% reduction in plating efficiency of BALB/c-3T3 cells. Following treatment with chemical agents, the cultures will be washed and incubated for an additional 24 hours in control medium. The cells will be infected with SV_{40} and the population passaged at several cell densities. Transformed

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clones will be scored in 10-14 days, and the enhancement of transformation by chemical plus virus treatment over that observed with chemical or virus alone will be determined.

To examine whether chemical agents can influence the outcome of viral transformation, growing cells will be infected with SV_{40}, and these cells will be exposed to chemical agents 24 hours after virus infection. Carcinogens and mutagens will be added to cultures for periods up to 48 hours at concentrations as described above. Co-carcinogens will be used throughout the entire interval of cell growth until the plates are stained for scoring of transformed clones.

Our recent work with early passage rat embryo fibroblasts and alkylating agent carcinogens, such as diepoxybutane, indicates that early chromosomal lesions occur as a result of such treatment (S.R. Wolman, A. Sivak and M.LaRocca, Proc. Am. Assoc. Cancer Res., 14: 38, 1973). Exploratory studies with a few selected chemicals and SV_{40} transformation will be carried out with these rat cells derived from Fisher 344 rat embryos.

Transformed clones from BALB/c-3T3 and Fisher 344 rat cell studies will be picked and tested for oncogenicity by injection of cells subcutaneously into newborn littermates.

9.b.2. BALB/c-3T3 Cells + Mouse Sarcoma Virus (MSV)

The focus assay system described by O'Connor (Perspective in Leukemia, p. 64, 1968, Grune and Stratton, N.Y.) will be employed with BALB/c-3T3 cells and fully competent MSV. The cultures of BALB/c-3T3 cells will be prepared (4×10^5 cells/50 cm dish) in Dulbecco's modification of Eagle's medium with 10 percent fetal calf serum. After 18-20 hours, the plates are drained and virus incubation carried out according to O'Connor. The treatment of cultures with chemicals prior to and after viral infection will be carried out according to the protocol described in 9.b.1. Cultures pretreated with chemical agents for 24 or 48 hours will be adjusted in cell number at the time of passage so that, at the time of virus inoculation, the plates will contain approximately 4×10^5 cells.

Foci of transformed cells will be counted 6-8 days after introduction of virus into the cultures.

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clones will be scored in [illegible] and [illegible] of tumor.

Since Freeman and his associates (Proc. Nat. Acad. Sci., 68: 445, 1971; J. Nat. Cancer Inst., 44: 65, 1970) are engaged in the examination of the effects of chemical carcinogens on rat cells chronically infected with Rauscher leukemia virus (Fl15 line), no significant effort with this system is planned. However, it appears useful to test a few selected compounds in both RNA virus systems. Of special interest would be a determination of the effects of tumor promoters on the Fl15 line of rat cells.

9.b.3. Hamster Embryo Cells + Herpes Simplex Virus, Type 2 (HSV).

A procedure for the demonstration of transformation by HSV has been reported by Duff and Rapp (J. Virol., 8: 469, 1971) and will serve as a model for these studies. This system, in contrast to the transformation of BALB/c-3T3 cells by SV_{40} or MSV, requires virus inactivated for its cytopathic effect but retaining oncogenic potential.

Primary hamster embryo cultures from inbred hamsters will be prepared according to standard procedures used in this laboratory (A. Sivak and B.L. Van Duuren, Exptl. Cell. Res., 49: 572, 1968). Secondary cultures will be prepared in Dulbecco's modification of Eagle's medium. The cell layers will be exposed to UV-irradiated HSV, and the cells propagated according to Duff and Rapp, except that plastic Petri dishes will be used throughout.

Treatment of cultures with chemicals will be as described in 9.b.1. except that post-infection treatment with carcinogens may not extend to the 3-4 weeks required for demonstration of transformed clones.

Representive transformed clones will be picked, propogated and tested for malignancy in newborn hamsters.

9.c. Assessment of Genetic Lesions

9.c.1. Cytogenetic Studies

The cell populations employed in this study will be examined for chromosomal alterations after treatment with the various chemicals.

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Cell cultures will be harvested 24 or 48 hours after passage or renewal of the medium to maximize the population of dividing cells. After a 3 hour exposure to Velban (0.1 µg/ml), the cells will be harvested by trypsinzation, swelled in 0.38% KCl, fixed in Carnoy's solution and air-dried on slides.

Cells in metaphase from each preparation will be examined after staining with acetoorcein. Whenever possible at least 50 cells per culture will be counted and analyzed. (Slides are scanned at 10X, and cells which are deemed suitable are analyzed under oil immersion (100X) and, if necessary, photographed and karyotyped). Metaphase cells will be scored for chromosome number and for the presence of abnormalities. Gaps*, breaks, deletions and exchange figures will be recorded. Other features affecting the whole of the nuclear material such as pulverization or centromeric splitting will also be recorded. In accordance with the Cytogenetic Standardization Commission of the Environment Mutagen Society, an achromatic area more than a chromatid width will be scored as a break. Results will be tabulated in terms of the number of aberrations per cell in each population and the percentage of aberrant cells per population.. (*Gaps, although recorded will not be included as aberrations).

9.c.2. Biochemical Studies

To analyze for chemically-induced genetic lesions at the molecular level, alkaline sucrose gradient analysis will be performed on H^3-thymidine labeled DNA from treated cells. This procedure is a sensitive assay for single strand breaks in DNA. Cells will be exposed to H^3-thymidine for 2 generations, and the cell density will be adjusted to be identical to the conditions for the chemical + virus studies. After treatment of the labeled cells with chemical at doses and for times used in the transformation assays, the cells will be harvested mechanically (A. Sivak, F. Ray and B.L. Van Duuren, Cancer Res., 29: 624, 1969). Aliquots of washed cell suspensions will be placed on gradients of alkaline sucrose and the DNA sedimented (R.A. McGrath and R.W. Williams, Nature, 212: 534, 1966; J.T. Lett, et al., Nature, 214: 790, 1967).

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The gradients will be fractionated by a Buchler Densi-Flow Gradient Sampler, and the radioactivity determined as trichloroacetic acid precipitates collected on Whatman GF-1 glass fiber filters.

This procedure together with the cytogenetic analysis will provide evidence as to whether chemical carcinogens influence viral transformation of mammalian cells because of a direct effect on the genetic material.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The work will be carried out at the A.J. Lanza Laboratories of the Institute of Environmental Medicine, Long Meadow Road, Tuxedo, New York in a laboratory outfitted for tissue culture and biochemical studies. For tissue culture several incubators, sterile box and water-bath are available. Spectrophotometric analyses will be carried out using either Cary or Hitachi UV-visible spectrophotometers; infrared analyses will be performed on Perkin Elmer infrared spectrophotometers. Clinical (International) and high speed (Sorvall SS-1) centrifuges are available for cell fractionation. A Gilson Linear Fractionator with an Ultraviolet Absorption Meter will be used to collect the gradients. There is access to a Model L2-Spinco centrifuge, a Nuclear-Chicago Mark I liquid scintillation spectrometer and a walk-in cold room. Microscopy will be performed using a Nikon Model M inverted microscope with a Sage Instruments, Inc., Model 500 cinephotomicrographic apparatus. Animal facilities exceed the requirements set forth by the Institute of Laboratory Clinical Resources.

11. Additional facilities required:

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12. Biographical Sketches:-

Name:- Andrew Sivak

Title:- Associate Professor

Birthdate:- REDACTED

Place of Birth:- REDACTED

Major Research Interest:- Mechanisms of Carcinogenesis

Research Experience

Rutgers University, Department of Bacteriology, Research Fellow - In vitro cultivation of Trypanosoma lewisi and effect of antibiotics on T. lewisi in the rat and in vitro. 1956-1957.

Rutgers University, Institute of Microbiology, Research Fellow Tracer and inhibitor studies leading to a partial elucidation of the route of biosynthesis of actinomycin and studies of the pathways of tryptophan metabolism in Streptomyces antibioticus. 1957-1960

Organisch-Chemisches Institute, University of Vienna, Austria. Post-Doctoral Fellow - Studied pathway of inositol catabolism in Schwanniomyces occidentails and the inducible enzyme which was responsible for the first oxidative step. 1960-1961

A.D. Little, Inc., Cambridge, Mass., Senior Biochemist - Devised analytical methods for the estimation of experimental cancer chemotherapeutic agents. Physiological distribution and toxicity studies of these agents in mice, rats, dogs, and monkeys. Mode of anti-tumor activity studies in mice bearing ascitic leukemia and in in vitro systems derived from these neoplasms. 1961-1963

Biodynamics, Inc., East Millstone, N.J., Director of Research for a consulting laboratory in the biological sciences. Scientific direction of biological and biochemical work dealing with studies in microbiology, physiology, biochemistry and toxicology. 1963-1964

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R: REDACTED MATERIAL

N.Y.U. Medical Center, Institute of Environmental Medicine. Associate Research Scientist, 1964-1968; Assistant Professor, 1968-1971; Associate Professor, 1971 - Study of cell membrane and control of cell division; mechanisms of tumor promotion in cell culture and animal systems; tumor promoting properties of cigarette tar; neoplastic transformation in cell culture systems; cytogenetic studies with chemical carcinogens and chromosome structure.

Other Experience

1952 - 1955	U.S. Navy, Commissioned Officer.
1959	Oak Ridge Institute of Nuclear Studies - Isotopes techniques training.

Education

B.S.	Rutgers Univ., New Brunswick, N.J.	1952	Biology
M.S.	Rutgers Univ., New Brunswick, N.J.	1957	Bacteriology
Ph.D.	Rutgers Univ., New Brunswick, N.J.	1960	Microbiol. - Biochem.
U.S.P.H.S.	Post-Doct. Fellow Organisch-Chemisches Inst., Univ. of Vienna, Austria	1960-1961	

Professional Societies

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N.I.H. Medical Scholar, Institute of Environmental Medicine, 1965-1966; Associate Research Scientist, 1966-1967; Assistant Professor, 1967-1971; Associate Professor, 1971 - Study of cell membrane [illegible] cell culture systems, mechanisms of tumor promotion in cell culture and animal systems.

13. Publications

A. Sivak and B.L. Van Duuren, Phenotypic expression of transformation: Induction in cell culture by a phorbol ester. Science, 157: 1443-1444 (1967).

A. Sivak and B.L. Van Duuren, A cell culture system for the assessment of tumor-promoting activity. J. Nat. Cancer Inst., 44: 1091-1097 (1970).

A. Sivak and B.L. Van Duuren, Cellular interactions of phorbol myristate acetate in tumor promotion. Chem.-Biol. Interactions, 3: 401-411 (1971).

A. Sivak and S.R. Wolman, Classification of cell types: Agglutination and chromosomal properties. In Vitro, 8: 1-6 (1972).

A. Sivak, Induction of cell division. Role of cell membrane sites. J. Cell. Physiol., 80: 167-174 (1972).

American Association for Cancer Research

Environmental Mutagen Society

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14. First year budget:

A. Salaries (give names or state "to be recruited").	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Andrew Sivak, Principal Investigator	R	-
Cell Biologist-Virology (Assoc. Res. Scientist) to be recruited	R	-
Technical		
Susan Kulina (Research Technican)		REDACTED
Animal Caretaker		
Fringe Benefits (14% S & W)		REDACTED

Sub-Total for A $ 31,037.

B. Consumable supplies (by major categories)

Glassware, Tissue Culture Vessels	$ 2,500.
Medium components, serum, biologicals	2,500.
Chemicals, Isotope labeled biochemicals	2,000.
Liquid nitrogen, carbon dioxide	1,200.
Mice, rats hamsters, animal food and bedding	2,000.

Sub-Total for B $ 10,200.

C. Other expenses (itemize)

Domestic travel- scientific meetings	$ 600.
Foreign travel - International Cancer Congress Florence, Italy, October, 1974.	700.
MS preparation	500.
Books and Reprints	300.

Sub-Total for C $ 2,100.

Running Total of A + B + C $ 43,337.

D. Permanent equipment (itemize)

Biohazard Hood	$ 3,000.
Tissue Culture Incubator	2,000.

Sub-Total for D $ 5,000.

E $ 6,501.

E. Indirect costs (15% of A+B+C)

Total request $ 54,838.

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$33,657.	$11,000.	$2,000.	-	$6,999.	$53,656.
Year 3	$36,181.	$12,000.	$2,000.		$7,527.	$57,708.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Principal Investigator			
Neoplastic Growth and Cell Cycle Control	NIH - CA-12503	$25,048.	5/1/73 to 4/30/74
Co-Investigator			
Initiation and Promotion in Carcinogenesis	NIH - CA-08580	38,396.	1/1/73 to 12/31/73
Tobacco Carcinogenesis	NIH - N01-CP-3-3241	84,620.	12/29/72 to 12/28/73
Carcinogen Induced Chromosome Changes and Transformation	NIH - CA-13821	23,871.	9/1/72 to 8/31/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Neoplastic Growth and Cell Cycle Control	NIH - CA-12503	Renewal	5/1/74 - 4/30/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy, Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Andrew Sivak

Signature Andrew Sivak Date 7/25/73

Telephone 212-679-3200 (Area Code) 5-228 (Number Extension)

Checks payable to

New York University Medical Center

Mailing address for checks

550 First Ave., New York, N.Y. 10016

Atten. John Ballow, V.P. For Finance

Responsible officer of institution

Typed Name Peter Arakelian

Title Vice President for Facilities and Services

Signature [illegible] Date 7/26/73

Telephone 212-679-3200 (Area Code) 2033 (Number Extension)

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#934 - STANLEY

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

August 6, 1973

Grant application #934
CARCINOGENESIS

To: The committee comprising Drs. Andervont, Huebner and Jacobson

Subject: Wendell M. Stanley, Jr., B.A., Ph.D., University of Calif., Irvine
New application #934
". . .Lethal Mutants of the C-Type Avian Myeloblastosis Virus"

History

This proposal originally was case #189. The mail ballot from the SAB Executive Committee was inconclusive, and several questions were asked. By the time answers were forthcoming, the July closing loomed, and Dr. Stanley was offered the option to apply "at his own risk", without preliminary evaluation as to program relevance.

Application #934 requests $65,420. direct costs plus two additional years.

Documents Submitted

Attached is application dated July 16, 1973.

Reprints or manuscripts of pertinent papers by the applicant have been provided, and will be forwarded if you wish.

Comment

As Dr. Stanley's letter dated May 21, 1972, concerning his original "case" defends relevance to our program objectives and answers other questions, a copy is appended.

FWN

F.W.N.

FWN:gh

Enclosure

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8. Brief statement of working hypothesis: Genetic and biochemical analyses of temperature sensitive and conditional lethal mutants of RNA-containing viruses which replicate in eukaryotic cells have proven to be extremely powerful methods for the elucidation of the molecular basis of the replication and of the disease-causing properties of such viruses. AMV is a typical member of the C-type RNA-containing tumor viruses which have been demonstrated to be associated with such human neoplastic diseases as rhabdomyosarcomas, breast cancers, various sarcomas and lymphomas, and leukemia. No temperature sensitive or conditional lethal mutants of AMV have ever been isolated. This is due to the fact that no suitable screening or assay system was available. This difficulty has now been overcome through the development by Dr. Thomas Graf of an avian RNA tumor virus plaque assay which can be used both at low and high (permissive and non-permissive) temperatures. The choice of AMV for this research proposal is based upon several important considerations. (1) AMV is a C-type virus which reproducibly induces leukemia. (2) The host range of AMV is extremely restricted; only chicken, quail, and turkey cells are affected. This ensures the safety of laboratory personnel. (3) AMV and AMV-producing cells (myeloblasts) can be obtained in very high titers and quantities from the blood of infected chicks. (4) AMV-producing myeloblasts can be grown in large quantities in tissue culture for extremely long periods of time with continual yields of high titers of AMV. This combination of the properties of AMV and of the cells in which it replicates makes AMV an ideal C-type virus for the broad investigation which I am proposing. No other C-type virus shares all of these advantages so necessary for my approach to the problem.

9. Details of experimental design and procedures (append extra pages as necessary)

Please see the appended pages. The research proposal and supporting information are organized as follows:

1. AIM AND METHOD OF STUDY

Specific aims (a more detailed explanation than allowed under Section 7)

Plan of attack
- Infected chick myeloblasts producing AMV
- Isolation of AMV virions
- Radioactively labeled AMV virions
- Temperature sensitive and conditional lethal mutants of AMV
- Isolation of high molecular weight AMV RNA
- Eukaryotic in vitro amino acid incorporation systems
- Characterization of products synthesized in vitro under the direction of wild-type and mutant AMV RNA

The significance of the studies proposed

2. WORK ACCOMPLISHED BY ME
- Unpublished results
- Published results

WORK ACCOMPLISHED BY OTHERS
- Temperature sensitive and conditional lethal mutants of RNA-containing eukaryotic viruses
- AMV RNA as mRNA
- Virion-associated components and virus-specific proteins as possible AMV RNA coded products

References

Source: https://www.industrydocuments.ucsf.edu/docs/nrcm0000

1. AIM AND METHOD OF STUDY

Specific aims: Using the recently developed plaque assay of Graf (1) for avian leukosis viruses, temperature sensitive and conditional lethal mutants of the oncogenic C-type avian myeloblastosis virus (AMV) will be sought. The mutants will be characterized by measuring the uptake in tissue culture of radioactive precursors into the proteins, glycoproteins, glycolipids, lipids, RNA, and DNA of AMV virions at both the permissive and nonpermissive temperatures. These techniques will allow the classification of AMV temperature sensitive and conditional lethal mutants into separate groups defective (at the nonpermissive temperature) in the biosynthesis of the several classes of macromolecules. Pairs of independently isolated mutants will be grown together at the nonpermissive temperature under various conditions in order to determine recombination and complementation groups. The high molecular weight RNA from wild-type AMV and from individual members of the various classes of mutant virions will be isolated and used as mRNAs in highly purified, initiation factor- and mRNA-dependent in vitro amino acid incorporation systems derived from several eukaryotes (2,3). The products synthesized at both the permissive and the nonpermissive temperatures under the direction of wild-type AMV RNA and of temperature sensitive and conditional lethal mutant AMV RNA will be compared by numerous techniques in order to identify and characterize the gene product corresponding to the mutation in the AMV genome. Analyses of the defects in macromolecular biosynthesis studied in tissue culture, the determination of recombination and complementation groups, and analyses of the products synthesized in vitro under the direction of wild-type and mutant AMV RNA should provide considerable knowledge of the mechanisms of virus-induced cell transformation, viral replication, and lead to a solution of the problem of which of the AMV RNA gene products is responsible for the induction, maintenance, and proliferation of the virus-induced neoplastic disease.

Plan of attack: Although temperature sensitive and conditional lethal of RNA-containing eukaryotic viruses have been exploited for only 3 or 4 years, they have proven to be extremely valuble tools for the investigation of virus-mediated cell transformation and of viral replication. No temperature sensitive or conditional lethal mutants of AMV have ever been isolated because of the lack of a suitable assay and screening system. The plaque assay developed recently by Graf (1) for avian leukosis viruses now makes the isolation of such mutants possible. Graf's assay is suitable for avian leukosis virues of subgroups B and D (1); AMV is known to consist predominantly of viruses of subgroup B (4). AMV has a very restricted host range, being confined to chickens, Japanese quail, and turkeys (5). This is one reason that I have chosen to work with this C-type virus; my laboratory does not have the complex safety facilities required for experimentation with viruses which can transform, and/or replicate in, mammalian cells. A second reason for the choice of AMV is that the virus accumulates in extremely high titers both in the plasma of sick chicks (6-8) and in tissue culture media (6,9). These properties, which other C-type viruses do not share, make the growth of large quantities of the virus and the purification of the viral RNA in mg quantities both safe and possible.

Infected chick myeloblasts producing AMV. Chicks (strain Shaver Starcross), younger than 24 hours post-hatching, will be inoculated interperitoneally with 5×10^9 AMV virions (BAI strain A, predominantly subgroup B) per

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chick in 0.5 ml of phosphate buffered saline; this insures the development of myeloblastosis in greater than 75% of the recipient chicks within 3 weeks (6). Blood smears from each chick will be inspected every few days starting 14 days after inoculation. When the myeloblast count in the blood reaches greater than 1.5×10^9 myeloblasts per ml of blood (equivalent to 25-40% of the blood volume), the chicks will be bled by cardiac puncture. The blood will be mixed immediately with heparin and chilled. After washing the myeloblasts by gentle centrifugation, the cells will be maintained in tissue culture (6,9).

Isolation of AMV virions. Bulk preparations of unlabeled wild-type AMV will be obtained from the plasma of chicks which show high titers of myeloblasts in blood smears 14-21 days following inoculation. Such plasma routinely contain greater than 5×10^{11} AMV virions per ml, and the isolation techniques for highly infectious AMV from such material are already standard operations in the laboratory (6). Mutant strains of AMV which have been selected for further study will be grown at 36° in tissue culture using bone marrow cells from healthy chickens and/or cells from chick embryos (see below). As in the case of AMV grown in tissue culture for the production of radioactively labeled AMV (see below), the tissue culture media will be harvested at 24 hour intervals and the AMV-producing cells returned to tissue culture with fresh media. This process will continue until sufficient material (approximately 10^{13} AMV virions) is accumulated to allow the isolation of about 0.1 mg of high molecular weight AMV RNA (6). The titer of AMV virions in tissue culture media, harvested every 24 hours, is in the range of $3\text{-}5 \times 10^9$ AMV per ml (6). AMV-producing cells may be kept in tissue culture for longer than 6 months while virus production continues undiminished (9). Mutant and radioactively labeled AMV virions will be isolated from tissue culture media pooled from repetitive harvests by techniques already developed in the laboratory (6). The virions will be stored under the conditions described by Smida and Smidova (10).

Radioactively labeled AMV virions. For the production of labeled virions, the tissue culture medium will contain appropriate radioactive precursors to protein, glycoprotein, glycolipid, lipid, RNA, and/or DNA. The medium, containing newly released AMV virions, will be separated from the myeloblasts or from the bone marrow cells from healthy chickens and/or cells from chick embryos by low speed centrifugation under sterile conditions every 24 hours. The AMV-containing medium will be saved, and the AMV-producing cells will be returned to tissue culture with fresh medium and radioactive label(s) for subsequent harvesting of radioactive AMV virions at periodic intervals. The media from repetitive harvests will be pooled and the AMV virions isolated as described above.

Temperature sensitive and conditional lethal mutants of AMV. For the induction of temperature sensitive and conditional lethal mutants of AMV, the AMV-producing infected chick myeloblast cultures will contain in the medium those mutagens (azacytidine, fluorouracil, nitrosoguanidine, etc.) which already have proven effective in generating temperature sensitive and conditional lethal mutants in other eukaryotic RNA-containing viruses. Isolated, purified, and concentrated AMV virions (see above) will be treated in vitro with proven and effective mutagenic agents (γ rays, ultraviolet light, nitrous acid, etc.). The dose of each mutagen or mutagenic agent will be regulated so as to give approximately a 5% survival of the AMV population. Untreated AMV controls and the mutagenized AMV stocks will be assayed by the use of the plaque assay technique of Graf (1). Individual plaques arising at 36° (chosen as the permissive temperature) from the mutagenized AMV stocks or from

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the mutagenized AMV-producing myeloblast cultures will be reassayed at 41° (chosen as the nonpermissive temperature). AMV will be recovered from individual plaques produced at 36° and then replaqued at 41°. If the titer of recoverable AMV virions from the original 36° plaque is too low to be assayed accurately at 41°, the cells and the AMV from the plaque will be subcultured at 36° in the presence of additional cells from healthy chicken bone marrow and/or from chick embryos. AMV virions will be recovered from these cultures, concentrated, and then plaque assayed at 41°. AMV from individual plaques produced at 36° which do not produce plaques at 41° will be selected and replaqued on multiple plates at 36° at high dilution. The material in the rare single plaques will be recovered and the AMV contained in the region of such plaques, theoretically representing a homogeneous population, will be grown as described under Isolation of AMV virions to yield mutant AMV stocks with a sufficient number of virions to enable high molecular weight AMV RNA preparations to be made (see below). Large numbers of each mutant will be plaqued at 41° in order to determine the reversion rate of each temperature sensitive and conditional lethal mutant. Only those mutant strains which exhibit genetic stability will be used for further studies.

The genetic defects in the AMV temperature sensitive and conditional lethal mutants will be characterized in tissue culture using bone marrow cells from healthy chickens and/or cells from chick embryos by measuring the uptake of radioactive precursors into proteins, glycoproteins, glycolipids, lipids, RNA and/or DNA of the AMV virions and into virus specific products at both the permissive and nonpermissive temperatures. Culture media will be separated periodically from the cells and the AMV virions in the media will be purified, concentrated, and assayed by counting plaques formed at 36° and 41°, by inoculation into newly hatched chicks (normal temperature = 41°), and by physical techniques such as equilibrium centrifugation in preformed sucrose density gradients. Material banding at the bouyant density of AMV (1.16-1.18 g per ml) will be analyzed by the presence of radioactivity in protein, and/or glycoprotein and/or glycolipid, and/or lipid and/or RNA and/or DNA, by the level of ATPase activity, and by quantitative electron microscopy in order to determine the extent of formation of non-infectious AMV virions. These techniques will allow the classification of the AMV temperature sensitive and conditional lethal mutants into separate groups defective (at the nonpermissive temperature) in the synthesis of the several classes of macromolecules. Pairs of independently isolated mutant strains of AMV will be inoculated into newly hatched chicks, assayed together at high multiplicities of infection by Graf's (1) plaque assay at 41°, and cultured together at high multiplicities of infection at 41° with bone marrow cells from healthy chickens and/or with chick embryo cells in order to determine recombination and recombination groups.

Isolation of high molecular weight AMV RNA. Isolated, purified, and concentrated AMV virions will be disrupted with dithiothreitol followed immediately by the addition of sodium dodecyl sulfate and phenol extraction at pH 9 to protect (11) the poly A-rich regions in the high molecular weight AMV RNA and to insure that the RNA is extracted efficiently into the aqueous phase (6,12). Chicken liver tRNA, isolated and highly purified in this laboratory, will be used as a carrier and protective agent during the isolation of the AMV RNA (6). The RNA in the aqueous phase following phenol extraction will be extracted with diethyl ether to remove residual phenol and then precipitated with 2 volumes of 95% ethanol at -20°.

1½% of the high molecular weight AMV RNA is known to contain several

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poly A-rich regions ranging in lengths between 100 and 200 nucleotides (13-15). Such poly A-rich regions exist in all eukaryotic mRNAs, and in all eukaryotic viral mRNAs including the RNA contained in eukaryotic C-type viruses; the only exceptions are the mRNAs for histones, which have uniquely short half-lives (16). The AMV RNA preparations, containing the added chicken liver tRNA, will be dissolved in 10 mM Tris-HCl (pH 7.6 at 20°), 0.2 mM $MgCl_2$, 0.5 M KCl and applied at 23° to columns of microcrystalline cellulose (17). These columns will be washed with the application buffer; the low molecular weight RNA components endogenous in AMV RNA preparations and the added chicken liver tRNA are not retained by the columns (6). Undegraded 70S AMV RNA will then be eluted from the columns by reducing the KCl concentration in the buffer from 0.5 M to 0.1 M (6). Zone velocity centrifugation of such AMV RNA preparations, either unlabeled or labeled, through preformed density gradients of ribonuclease-free sucrose indicates that this material represents undegraded 70S high molecular weight AMV RNA in pure form (6).

Eukaryotic in vitro amino acid incorporation systems. The control (wild-type) and the various mutant AMV RNA preparations will be used as mRNAs at both the permissive and nonpermissive temperatures in highly efficient in vitro amino acid incorporation systems developed in this laboratory (2,3,6). These systems are derived from eukaryotic sources such as rabbit liver, rabbit reticulocytes, chicken liver, chicken reticulocytes, AMV-producing chick myeloblasts, 11 to 14-day old chick embryo leg muscles, and rat liver. All of these systems can be made completely dependent upon several exogenous protein synthesis initiation factors (2,3,18-21), tRNA (2,3), and mRNA (2,3,6). Homologous preacylated tRNA is used in each system, and each system has been shown to translate properly both the α and the β rabbit globin mRNAs (2,3).

The availability of heterologous in vitro amino acid incorporation systems is of paramount importance since group-specific antigens corresponding to the avian leukosis-sarcoma complex are found in "leukosis-free" chick embryos (22-25); this property of chickens is inherited as a single Mendelian dominant gene (26), and bears a striking resemblance to a similar situation found in mice which can synthesize group-specific antigens while exhibiting no detectable complete replicating leukemia viruses (27). These phenomena are consistent with the "virogene" and "oncogene" concepts of Todaro and Huebner (28), as are the AMV RNA-host cell DNA annealing data of Baluda and coworkers (29) which point to the presence of AMV genes in the DNA of both phenotypically normal and AMV-infected chicken cells.

Characterization of products synthesized in vitro under the direction of wild-type and mutant AMV RNA. Radioactive products synthesized in vitro at both the permissive and nonpermissive temperatures under the direction of wild-type AMV RNA or of mutant AMV RNA will be mixed in independent experiments with components obtained from wild-type AMV virions and with components from the mutant AMV virions from which the mutant RNA was extracted. In most cases, the AMV virions will have been radioactively labeled in tissue culture; however, some AMV virion preparations will be labeled in vitro, either before or after disruption of the virions with Triton X-100, with ^{125}I using the lactoperoxidase iodination reaction which specifically labels histidine and tyrosine residues (30-33). The ribonucleoprotein cores from AMV virions will be isolated by the method of Stromberg (34) using Sterox SL; in most experiments the components of the cores will be labeled in tissue culture, but for some experiments the proteins of the cores will be labeled in vitro with ^{125}I as described above.

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Many of the techniques for the identification and/or purification and/or measurement of C-type viral group-specific and type-specific antigens and of enzymatic activities will be discussed under WORK ACCOMPLISHED BY OTHERS, subsection Virion-associated components as possible AMV RNA coded products. Additional methods which will be used will include affinity chromatography of viral glycoproteins (35) and direct radioimmunoassay of group-specific antigens (a technique which is 100-fold more sensitive than complement fixation and 1,000-fold more sensitive than gel immunodiffusion) (36). Group-specific antigens will also be detected by the double antibody method (37), by immunoelectrophoresis (38), by N-terminal amino acid sequence data (39,40), and by isoelectric focusing (41). Antisera containing antibodies directed against purified AMV components will be produced by standard techniques in hamsters, pigeons, rabbits, rats, or guinea pigs. Also to be used for the fractionation of AMV components will be the standard techniques of ion exchange chromatography upon modified celluloses and hydroxyapatite, polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, and gel filtration through Sephadex and through Sepharose in the presence and absence of denaturing agents such as urea, acetic and formic acid, sodium dodecyl sulfate, and guanidine hydrochloride. All AMV component fractionation experiments will be of the double-label variety; one radioactive label (^{3}H, ^{14}C, or ^{35}S) will be incorporated into the products synthesized in one of the in vitro amino acid incorporating systems under the direction of the RNA extracted from either the wild-type AMV or from one of the AMV temperature sensitive or conditional lethal mutants and the second radioactive label (^{3}H, ^{14}C, ^{35}S, or ^{125}I) will be contributed by viral components from either wild-type AMV virions or from temperature sensitive or conditional lethal mutant virions of AMV grown in tissue culture or by wild-type or mutant viral components labeled in vitro. Correspondence between the 2 radioactive labels in protein will be confirmed by subjecting the material to digestion with trypsin, chymotrypsin, subtilisin, papain, pepsin, or pronase, followed by analysis of the resultant peptides by 2 dimensional paper electrophoresis and chromatography (42) and autoradiography, and by gradient elution chromatography upon columns of Dowex 50 (43).

The significance of the studies proposed: About 5 years ago, a line of cells (RD cells), which neither contained nor produced C-type particles, was established in tissue culture (44). These cells, originally obtained from a human rhabdomyosarcoma, were inoculated prenatally into 4 kittens and subsequently produced disseminated rhabdomyosarcomas consisting of cells with a human karyotype (45). 2 of the cat tumors (1 of which was a brain tumor) and a tissue culture line (RD-114; a mixture of 2 morphologically distinguishable cell types) established from the brain tumor did, however, produce a C-type virus (RD-114). Several lines of evidence indicate that the RD-114 virus may be of human origin. The cells from which the virus buds have a human, rather than a feline, karyotype; the viral components cross-react with the #3 group-specific (inter-species-specific) antigens of C-type viruses from numerous sources, but the #1 group-specific (species-specific) antigen does not react in complement fixation tests with antisera to the #1 group-specific antigens of feline, murine, hamster, rat, avian (46), or Russell's viper (47) C-type viruses. It is of interest that the #1 and the #3 group-specific antigens are associated with the same protein located in the internal ribonucleoprotein core of the virus (48). RD-114 does not interfere with the transforming properties of feline

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C-type viruses of subgroups A, B, or C; nor is it inactivated by antisera to feline C-type viruses (46). The RD-114 virus has the typical physical properties of a C-type particle: bouyant density in sucrose = 1.16-1.18 g per ml, contains 70S RNA and a RNA-dependent DNA polymerase, and is about 100 nm in diameter with C-type morphology (46). RD-114 does not transform any cell line tested (including human), but it will replicate in human and canine cells (46). The RNA-dependent DNA polymerase is distinguishable immunologically from the corresponding enzyme from C-type viruses from all sources including lower mammals and primates (49,50). More recent information concerning the origin of RD-114 indicates that cats may possess 2 distinct types of C-type viruses, and that RD-114 may belong to the newly discovered second type (51). McAllister et al. (51) have induced the formation of a C-type virus (CCC virus) from a continuous feline cell line (CCC) in the presence of iododeoxyuridine. The group-specific antigenic properties of this new virus are related to the corresponding antigen of RD-114. The new virus is able to replicate in RD cells and other human or monkey cells, but is unable to grow in feline cells (51). This new evidence lessens the probability that RD-114 is an etiological agent in human cancer.

Strong evidence that C-type viruses are associated with human breast cancer has been accumulated in recent years. The electron microscope has been used to detect C-type particles in human milk (52-55), in thin sections of breast tumor tissue and in pleural effusion cells from breast cancer patients (56). These particles possess the distinguishing features of C-type viruses (57-61), although they also are found in milk from apparently normal women (62) and epidemiological studies have failed to establish an etiological role for these virus particles in human breast cancer (63). On the other hand, C-type virions from human milk have been produced continuously for periods of time longer than a year by co-cultivation with human embryonic cells in tissue culture (64). It also is of significance that 70S RNA-containing C-type particles from extracts of malignant human breast adenocarcinomas (but not of normal breast or of benign breast fibroadenomas) can use their virion-associated RNA-dependent DNA polymerase to synthesize DNA which contains nucleotide base sequences which are complementary to the RNA from mouse mammary tumor virus (65). RNA obtained from human breast adenocarcinomas (but not RNA from normal breast tissue or from such benign pathologies as fibrocystic disease or fibroadenoma) also possesses base sequences homologous to the base sequences in mouse mammary tumor virus RNA (66), but not to the base sequences in Rauscher leukemia virus RNA (65). None of the C-type particles associated with human breast cancer has been shown to be infectious or transforming for human cells.

RNAs extracted from human leukemia cells, sarcomas, and lymphomas have been shown to have base sequences homologous to the RNA from Rauscher leukemia virus (67-69). C-type particles containing 70S RNA and an endogenous RNA-dependent DNA polymerase have been obtained from the peripheral white blood cells of patients suffering from acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelogenous leukemia. These C-type particles from human neoplastic cells or tissues are able to synthesize DNA with base sequences complementary to the RNA from Rauscher leukemia virus, but not to the RNA from mouse mammary tumor virus or to the RNA from AMV (70).

To summarize these results, the RNA in C-type viruses from human breast cancers is at least partially homologous with the RNA from a C-type virus known to induce mammary cancer in mice (mouse mammary tumor virus), but not

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to RNA from a known C-type leukemia virus (Rauscher leukemia virus). In contrast, the RNA in C-type viruses from human leukemia cells is at least partially homologous with the RNA from a C-type virus known to induce leukemia in mice (Rauscher leukemia virus), but not to RNA from a C-type virus known to cause mammary tumors in mice (mouse mammary tumor virus) or to the RNA from a C-type virus known to induce myeloblastic leukemia in chickens (AMV).

The analyses of AMV-specific macromolecular biosynthesis and the determination of recombination and complementation groups through the use of temperature sensitive and conditional lethal mutants of AMV, coupled with an identification and characterization of virion-associated and virus-specific products synthesized in vitro under the direction of the wild-type and of the mutant AMV high molecular weight RNA genomes, will allow many insights into the mechanisms of C-type virus-induced cell transformation and viral replication. These approaches appear appropriate for an identification and characterization of the AMV gene product(s) which lead(s) to the induction, maintenance, and proliferation of the myeloblastic leukemia. Because the biochemistry and molecular biology of higher eukaryotic life forms are so similar, such results should have relevance to the control of neoplastic growths in man.

2. WORK ACCOMPLISHED BY ME

Unpublished results. A strain of AMV (BAI strain A, predominantly subgroup B) and a strain of White Leghorn chickens (Shaver Starcross) have been found which together yield a very high incidence of myeloblastosis in young chicks (6). Techniques have been developed for the isolation of the AMV virion both from plasma and from tissue culture media, thus allowing both the purification of unlabeled virus in very large quantities and radioactively labeled labeled virus in reasonable and workable quantities (6).

Extremely effective techniques have been modified from published methods for the isolation of the high molecular weight RNA genome of AMV. The adsorption to, and desorption from, microcrystalline cellulose of the AMV 70S RNA, dependent upon the presence of poly A-rich regions in the high molecular weight RNA, has proven to be particularly useful (6). Personnel in my laboratory also have used this method successfully for the purification and isolation of undegraded mRNAs for rabbit and chicken globins and for chick embryo myosin (3,6,71). Using poly A of known chain lengths (72), the chain length dependence of poly A retention by microcrystalline cellulose under standard conditions and the ionic strength dependence of such retention by poly A chains of known chain lengths were determined (6). At 23°, in 10 mM Tris-HCl (pH 7.6 at 20°), 0.2 mM $MgCl_2$, 0.5 M KCl, poly A chains of 50 or more residues are retained by microcrystalline cellulose. Chains of poly A containing more than 50 residues could be fractionated according to length by gradually reducing the ionic strength. Experiments with pure lignin have shown that the suggestion of Delarco and Guroff (73) that the adsorption of purine-rich regions of RNA to cellulose is due to a "lignin-like" contaminant is without merit (6).

Preliminary experiments, based upon the data relating ionic strength and the chain length of poly A retained by microcrystalline cellulose, have indicated that mRNA from chick embryo leg muscle polysomes (which contains the mRNA for myosin) can be clearly separated on columns of microcrystalline cellulose by a reverse salt gradient, and that the 4S and the 4-6 30-40S RNAs resulting from the denaturation of the 70S AMV RNA can be fractionated by essentially the same technique (6). This is an extremely important development,

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since even high resolution electrophoresis through polyacrylamide gels and chromatography upon modified celluloses or methylated albumin-coated kieselguhr have not been successful in this type of fractionation.

Chicken and rabbit globin mRNAs, chick myosin mRNA, and 70S AMV RNA have been used as mRNA in the in vitro eukaryotic amino acid incorporation systems available in my laboratory (2,3,6) with excellent results. Several variables which usually are not controlled well in in vitro systems have been found to be extremely important for the translation of natural and viral mRNAs, exclusive of the effects of mRNA-specific initiation factors. We have confirmed the observation of Olsnes et al. (74) that low levels of non-ionic detergents increase the efficiency of in vitro translation. Olsnes et al. used Triton X-100 and Brij-58 in a rabbit reticulocyte in vitro system which is practically devoid of membranous material; we have found that Nonidet P-40 is even more effective (6). The ionic concentration and composition of both mono- and divalent cations have been found to be critical during in vitro translation. For example, under certain conditions, only the mRNA for the rabbit β globin chain is translated, while under other conditions, the translation of the mRNA for the rabbit α globin chain is favored (3).

My laboratory has continued work on the identification, purification, and characterization of eukaryotic protein synthesis initiation factors (18-21). All of the data published from my laboratory has since been confirmed by workers in other laboratories (75-80). The results essentially are not in agreement, with the possible exception of "M_3", with the very numerous publications from the group working with Dr. W. F. Anderson beginning in late 1969 and continuing to the present.

We have several manuscripts in preparation which describe further the properties, functions, and improved purifications of 2 eukaryotic initiation factors (IF-I and IF-II) (71). IF-I is a large protein (molecular weight = 300,000), obtained from high salt washes of polysomes, which can be reversibly inactivated by sulfhydryl reagents. The first function of IF-I, which has been purified almost to homogeneity (about 500-fold relative to the polysomal wash), is to bind specifically the eukaryotic initiator tRNA (Met-$tRNA_i^{Met}$) in a GTP-dependent reaction; the formation of this ternary complex is AUG- and magnesium ion-independent, activated by thiols, and is exceptionally stable at physiological temperatures; its formation is blocked by very low concentrations of the initiation inhibitor aurintricarboxylate. The second function of IF-I is to bind the initiator tRNA, in an AUG- and mRNA-independent reaction, to the 40S ribosomal subunit. There is no tissue or species specificity in these reactions with respect to either the initiator tRNA or IF-I (tissues tested: rabbit liver, rabbit reticulocytes, chicken liver, chicken reticulocytes, chick embryo leg muscles, and rat liver) (21). The N-formylated derivative of the initiator tRNA, which is not a natural initiator tRNA in the cytoplasm of eukaryotes, binds to IF-I to only 1/10 the extent of the natural initiator tRNA (21). In the presence of IF-II, which also is a large protein (molecular weight = 400,000) sensitive to sulfhydryl reagents, and separable from IF-I by ion exchange chromatography on DEAE-cellulose, the initiator tRNA-40S ribosomal subunit complex is joined by a 60S ribosomal subunit to form an 80S initiation complex (81). This reaction is stimulated by the AUG codon, but does not require natural or viral mRNAs (81). We have developed 2 in vitro amino acid incorporation systems, both dependent upon exogenous mRNA, from rabbit reticulocytes by 2 different techniques. One system cannot translate the mRNA for rabbit α globin although large quantities of rabbit β globin are synthesized; the second system synthesizes both globin chains efficiently (3). We are now

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screening the second system for the α globin mRNA-specific IF-III initiation factor in order to assay it in the first in vitro system. This mRNA-specific initiation factor will be purified and its precise role in controlling the translation of a particular mRNA investigated. This will be the first demonstration of an mRNA-specific IF-III which absolutely regulates the translation of a specific mRNA.

We also are concentrating on developing an in vitro amino acid incorporation system, as efficient as the other eukaryotic systems used in the laboratory, from AMV-producing chick myeloblasts (6). We are isolating, purifying, and characterizing protein synthesis initiation factors from chick embryos, other chicken tissues, and AMV-producing chick myeloblasts. The complete translation of the AMV genome may well depend upon the availability of specific initiation factors which regulate the efficiency and temporal expression of the AMV RNA. When available, these specific initiation factors from chickens will be used in a heterologous in vitro amino acid incorporation system because of the possibility of endogenous synthesis of group-specific antigens and perhaps other products specified by C-type viral information inherited by the chick embryo, the chick, and the adult chicken.

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WORK ACCOMPLISHED BY OTHERS

Temperature sensitive and conditional lethal mutants of RNA-containing eukaryotic viruses. Temperature sensitive and conditional lethal mutants have been successfully isolated from stocks of Rous sarcoma virus (82-89), Sindbis virus (90), vesicular stomatitus virus (91), reovirus (92), and influenza virus (93). Studies upon some of the temperature sensitive mutants of Rous sarcoma virus have shown that the transformation of the infected cell is reversible, and that the maintenance of the transformed state of the cell requires the constant expression of a viral gene which is not required for virus multiplication (84-89,94). Other studies have demonstrated that the transformed cell morphology expressed at low temperatures (36°) is associated with a drop in the intracellular concentration of cyclic AMP (95). Lai and Duesberg (96) have investigated the glycoproteins and glycopeptides from temperature sensitive mutants of Rous sarcoma virus and find that the glycoproteins from the virion grown at the permissive temperature (36°) have lower electrophoretic mobilities than those from the virion grown at the nonpermissive temperature (41°). It also was reported that the glycopeptides derived from virions released from transformed cells possess higher molecular weights than those from virions released from phenotypically normal cells (96). Some temperature sensitive mutants of Rous sarcoma virus express their defects by altering cell morphology (82), while some conditional lethal mutants cannot replicate at 41°; these mutants may express their defects either early (ts 149) or late (ts 75) in the infectious process (97).

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Using conditional lethal mutants of vesicular stomatitus virus, Szilagi and Pringle (91) demonstrated that in the group I mutants the mutated gene is the structural gene for the virion-associated RNA-dependent RNA polymerase.

Analyses of conditional lethal mutants of reovirus have revealed 3 recombination and coincident complementation groups (C, D, and E) (98). All mutants in the 3 groups are defective in the synthesis of double-stranded RNA at the nonpermissive temperature (39°), although the infected cells continue single-stranded mRNA synthesis (98). Mutants falling in groups C and D abruptly shut off double-stranded RNA synthesis upon being shifted from 31° to 39°; mutants in group E continue double-stranded RNA synthesis at the normal rate following such a temperature shift (98). After a physical analysis of the RNA species synthesized at the 2 temperatures by the mutants, a model was proposed to explain the nature and consequences of the mutations (99). It was concluded that group D mutants are defective in the structural gene(s) for virion polypeptide chain(s) (100). Other workers have investigated conditional lethal mutations in 5 cistrons of reovirus (101). Different mutants produce different amounts of viral polypeptides, but all of the mutants are able to synthesize all of the major viral polypeptides as judged by molecular weights and immune precipitations, although these polypeptides cannot be assembled into a virion structure (101). Reovirus mutants also have been studied which are defective in the ability to cleave precursor viral polypeptides into the final virus-specific proteins (102). Mutants of Sindbis virus (103) and of polio virus (104) also have been isolated and found to be defective in the processing of precursor high molecular weight viral polypeptides.

No temperature sensitive or conditional lethal mutants of AMV have ever been isolated.

AMV RNA as mRNA. There is remarkably little information on the use of AMV RNA as mRNA in in vitro amino acid incorporation systems. The information summarized below, and the fact that high molecular weight AMV RNA, not phenol extracted and containing about 6% protein, has been reported to be infectious in young chicks (105) (a report that has not been extended or confirmed either by the original workers or by workers in other laboratories), strongly supports the idea that the RNA contained in the AMV virion is the plus strand; i. e., able to be translated correctly by polysomes to yield virus-specific products. AMV RNA has been used as mRNA in in vitro amino acid incorporation systems derived from Escherichia coli, a prokaryote (106-110). In the first 4 publications, no attempt was made to identify or to characterize the products made in vitro. These studies are therefore suspect, since it has been shown that the addition of high molecular weight RNA to an in vitro system can stimulate the synthesis of endogenous products without the exogenous RNA itself being translated (111). Siegert et al. (110) have made the only analyses of the products synthesized under the direction of AMV RNA in E. coli extracts; they were able to identify the #4 group-specific antigen immunologically and to make a preliminary identification of the #1, #2, and #3 group-specific antigens solely on the basis of molecular weights.

Gielkens et al. (112) have used Rauscher leukemia virus RNA and mouse mammary tumor virus RNA as mRNAs in an in vitro system from E. coli. The products made in vitro were co-electrophoresed with the respective virion proteins, but the results were not particularly convincing that virus-specific proteins had in fact been synthesized. Gielkens et al. (112) also mentioned that attempts to translate these viral RNAs in oocytes or in a rabbit reticulocyte in vitro system were unsuccessful. In contrast to these negative results, Vidrine et al. (113) have reported that Rauscher leukemia virus

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RNA is an effective mRNA in extracts of Rauscher leukemia virus-infected tissue cultured cells originally obtained from mice. However, no evidence for the synthesis of virus-specific products was presented. The same group of workers subsequently demonstrated that Rauscher leukemia virus RNA would enter into polysomes from Rauscher leukemia virus-infected cells in the presence of a high salt wash of homologous polysomes (114). Such washes of polysomes are known to contain, among other ribosomal proteins, initiation factors for protein biosynthesis.

There is no published information concerning the translation of AMV RNA in in vitro amino acid incorporation systems derived from eukaryotes.

Virion-associated components and virus-specific proteins as possible AMV RNA coded products. The proteins (115-117), glycoproteins (118-121), and, to a lesser extent, the glycolipids (120) and phospholipids (122) of the viruses comprising the avian leukosis-sarcoma complex have been fairly well characterized. Most attention has been focused on the type-specific antigens (located in or on the external envelope of the virion) (118-121, 123-132) and on the group-specific antigens (located in the internal ribonucleoprotein core) (119, 133-140). The nucleoprotein core itself has been isolated and extensively studied (34, 141-145).

There are, however, components associated with the AMV virion which are not as well known or as well characterized. The following entities and/or activities have been reported to be associated with the AMV virion: ATPase (146), RNAase (147-150), DNA-specific endonuclease (151,152), RNA methylase (153), RNA-dependent DNA polymerase and RNAase H in a single subunit (152,154-156), a protein that stimulates the RNA-dependent DNA polymerase (157), aminoacyl-tRNA synthetases (158), specific ribosomes (159) with their ribosomal RNA and approximately 63 individual proteins (160), 7S DNA (161,162), about 20% of the RNA content of AMV which represents 4S RNA which does not accept amino acids and 4S tRNA which accepts amino acids, is functional in protein synthesis and is derived, but distinct from, the host cell tRNA (163-172), and 4S tRNA-like RNA which is released from the high molecular weight AMV 70S RNA genome only under denaturing conditions (173).

In addition to most of the above components, the Rous sarcoma virion has been reported to be associated with DNA ligase (174), DNA-specific exonuclease (174), nucleotides (175), nucleotide kinase (175), phosphatase (175), hexokinase (175), and lactate dehydrogenase (175).

Finally, an RNA-dependent RNA polymerase, which prefers AMV RNA as a template, has been reported to exist in AMV-infected myeloblasts (176,177). However, there is disagreement concerning the intracellular presence of RNA complementary to the RNA in the AMV virion. Such complementary RNA has been reported to be located in the nucleus of infected myeloblasts (178), although Baluda and Nayak have not been able to detect any cellular RNA complementary to the virion RNA (179,180).

In view of the large number of virion-associated components and the possible existence of an intracellular enzyme in infected myeloblasts involved in the replication of AMV RNA through a complementary RNA template, it is important to determine which of these entities are coded for by the RNA genome of AMV.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space: Two-room laboratory (rooms 468 and 470A in the Department of Molecular Biology, School of Biological Sciences, Steinhaus Hall, University of California, Irvine, California 92664); total of 1074 square feet.

Community space: Cold rooms (-20° and +4°), warm rooms (25° and 37°), a dark room and autoradiography and photographic supplies, a high voltage electrophoresis explosion-proof room, an animal facility (UCI Vivarium), a room for chickens, and the UCI Media Kitchen with autoclaves.

Equipment: L3-50 Beckman Spinco ultracentrifuge, ultraviolet and visible spectrophotometers (Beckman DB, Perkin-Elmer Colleman Hitachi model 124, Gilford models 240 and 2000), RikaDenki single pen recorder, Mettler H-10 semi-micro balance, Buchler refrigerated fraction collector, 2 Isco model 272 fraction collectors, Forma model 2095-2 heating and cooling circulating water bath, 3 Napco model 220 water baths, Barnstead model EPR-½C redistiller, metering pumps (Buchler Polystaltic, Sigmamotor peristaltic, Stalprodukter peristaltic, Beckman piston metering pump), Thermovac model FD-1-V2DG lyophilizer, Sears and Roebuck egg incubator and chick brooder, ultraviolet-irradiated sterile box, refrigerators (Coldspot small and regular size and Glenco large stainless steel), -20° freezer, Kelvinator series 100 -76° freezer, Buchler flash evaporator with vacuum pump, Thelco model 29 vacuum oven, National 37° forced air incubator, gel electrophoresis supplies (includes de-stainer and gel scanner), and thin layer and column chromatography supplies.

Community equipment: Scotsman chipped ice machine, Gilson Electrophorator model D high voltage electrophoresis equipment, Beckman amino acid analyzer, electron microscope, 1 L2 and 2 L2-65B Beckman Spinco ultracentrifuges, polyacrylamide gel slicer, American Optical Spencer model 1036 microscope, and autoclaves.

11. Additional facilities required:

The facilities or equipment required to carry out the research proposed are requested and justified in the budget under Section 14. My most pressing needs are for a scintillation spectrometer, for increased centrifugation capacity, and the equipment to allow bulk growth of cells and AMV in tissue culture and to screen and assay for mutants of AMV at the permissive and nonpermissive temperatures (i. e., 2 water-jacketed CO_2 incubators). My preliminary experiments have been carried out with make-shift and inadequate facilities. As may be seen from the list of equipment that I already possess, the laboratory is set up well for biochemical and molecular biology studies, but not for large-scale virus production or for careful and extensive tissue culture studies.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12. Biographical sketch of principal investigator:

Name: Wendell M. Stanley, Jr.
Title: Associate professor, step II. 2/3 appointment in the Department of Molecular Biology and Biochemistry, and 1/3 appointment in the Department of Biological Chemistry, California College of Medicine, both on the Irvine campus.
Birthdate:
Place of birth: REDACTED
Nationality:
Sex: Male
Marital status:

REDACTED

Education:	Univ. of Cal., Berkeley	A.B.	1957	Chemistry & Biochem.
	Univ. of Wis., Madison	M.S.	1959	Biochemistry
	Univ. of Wis., Madison	Ph.D.	1963	Biochemistry
	N. Y. U. Sch. Med., N. Y.	U.S.P.H. postdoctoral fellow	1963-1965	Biochemistry

Employment: Instructor in Biochemistry, N. Y. U. Sch. Med., Dept. of Biochemistry, 1965-1966.
Assistant professor of Biochistry, N. Y. U. Sch. Med., Dept. of Biochemistry, 1966-1967.
Assistant professor of Biochemistry, Univ. of Cal., Irvine, Dept. of Molecular Biology and Biochemistry (2/3), and Dept. of Biological Chemistry, California College of Medicine (1/3), 1967-1970
Associate professor of Biochemistry, same as above, 1970-present.

Honors: Honors in Chemistry, Univ. of Cal., Berkeley, 1957.
Phi Beta Kappa, Univ. of Cal., Berkeley, 1957

Major research interest: Avian myeloblastosis virus, control of protein synthesis.

Role in proposed project: Principal investigator.

13. Publications (five most recent. Not included below are four manuscripts currently accepted for publication which are in press. A list of these may be found under WORK ACCOMPLISHED BY ME, Published results. A fifth manuscript will be communicated by Dr. Howard K. Schachman to the Proceedings of the National Academy of Sciences U. S. within the next week; Two Eukaryotic Protein Synthesis Initiation Factors (IF-I and IF-II) are Required to Form an Initiation Complex with Rabbit Reticulocyte Ribosomes, Cashion, L. M., Neal, P. M. and Stanley, W. M., Jr.)

1. An Economical Procedure for the Preparation of L-[^{35}S]Methionine of High Specific Activity, Graham, A. R. and Stanley, W. M., Jr., Anal. Biochem. 47 505 (1972).
2. Initiation of Protein Synthesis: The Interaction of Methionine-Specific Transfer Ribonucleic Acids with Rabbit Reticulocyte Ribosomes, Cashion, L. M., Kolb, A. J. and Stanley, W. M., Jr., Biochim. Biophys. Acta 262 525 (1972).
3. Recognition of Eukaryotic Initiator tRNA by an Initiation Factor and the Transfer of the Methionine Moeity into Peptide Linkage, Dettman, G. L. and Stanley, W. M., Jr., Biochim. Biophys. Acta 287 124 (1972).

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Name: Donald M. Stanley, Jr.

Title: Assistant Professor, Dept. [illegible]

4. Preparation and Analysis of L-[^{35}S]Methionine Labeled Transfer Ribonucleic Acids from Rabbit Liver, Stanley, W. M., Jr., Anal. Biochem. 48 202 (1972).

5. The Ternary Complex of Initiation Factor IF-I, Met-tRNA$_f^{Met}$ and GTP. An Aurintricarboxylate-Sensitive Intermediate in the Initiation of Eukaryotic Protein Syntheis, Dettman, G. L. and Stanley, W. M., Jr., Biochim. Biophys. Acta 299 142 (1973).

[illegible]

13. Publications (five most recent, not included above) or [illegible] manuscripts currently [illegible]

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	† Amount
Professional (give % time of investigator(s) even if no salary requested)		
Wendell M. Stanley, Jr. (Principal Investigator)		
(3/9 summer salary, 3 months)		
Linda M. Cashion (PhD expected Sept. 1973)		
Glenn E. Brown (PhD expected Sept. 1973)		
Alfred J. Kolb (graduate student)		REDACTED
Bryn Henderson (graduate student)		
Peter Neal (graduate student)		
Technical		
Marcia E. Witte (Lab. Asst. I, Step III)*		
*Reflects 5% merit + 6% cost of living		
Kent Cooper (Laboratory Helper)		REDACTED
Larry Dickinson (Laboratory Helper)		
Steve Noble (Laboratory Helper)		
Retirement and other security benefits (total)		1,049
†All salary and benefit rates are set by University policies.	Sub-Total for A	16,279
B. Consumable supplies (by major categories)		
Replacement drive for Spinco L3-50 ultracentrifuge		500
Tissue culture media and supplies		3,000
Chemicals and biochemicals		2,000
Radioactive materials		3,500
Animals and animal boarding in UCI Vivarium		6,000
Glass- and plasticware		1,000
Radioactive counting supplies		1,000
Radioactive waste disposal		250
+ 6% Calif. sales tax (not on animal boarding)		795
	Sub-Total for B	18,045
C. Other expenses (itemize)		
Service contract for Spinco L3-50 ultracentrifuge		300
Artwork, photography, page costs, reprints		1,000
Repairs and maintenance on laboratory equipment		1,250
	Sub-Total for C	2,550
	Running Total of A + B + C	36,874
D. Permanent equipment (itemize)		
Beckman LS-330 scintillation spectrometer		16,000
Leeds and Northrup 7415-E2 research pH meter		650
Amicon DC2 hollow fiber dialyzer/concentrator		850
CRC Labwasher + installation		1,780
New Brunswick T-41 bottle roller for tissue culture		2,400
Two New Brunswick CO-20 CO_2 incubators		2,600
Beckman Spinco type SW-40-Ti rotor		2,650
+ 6% Calif. sales tax		1,616
	Sub-Total for D	28,546
E. Indirect costs (15% of A+B+C)	E	5,531
	Total request	70,951

15. Estimated future requirements: Please see appended detailed future budget and justifications.

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	17,724	20,324	3,100	4,441	6,172	51,761
Year 3	18,486	23,709	3,650	8,295	6,877	61,017

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R: REDACTED MATERIAL

Budget:

	Year 1	Year 2	Year 3
Personnel† (For consultants, see page 10a.)			
W. M. Stanley, Jr. (Principal investigator) R	REDACTED		
REDACTED			
Marcia E. Witte (Lab. Asst. I, Step III) R	REDACTED		
Laboratory Helpers (2) (50%)			
Retirement or other security benefits (total)			
Category total	REDACTED		
Permanent equipment			
Beckman LS-330 scintillation spectrometer	16,000	0	0
Leeds and Northrup 7415-E2 research pH meter	650	0	0
Amicon DC2 hollow fiber dialyzer/concentrator	850	0	0
CRC Labwasher + installation	1,780	0	0
New Brunswick T-41 bottle roller for tissue culture	2,400	0	0
Two New Brunswick CO-20 CO_2 incubators	2,600	0	0
Spinco type SW-40-Ti rotor	2,650	0	0
Sorvall RC2-B centrifuge with SS-34, GSA, and GS-3 rotors	0	4,190	0
Van Waters & Rogers vacuum pump	0	0	800
Amicon MMC concentrator	0	0	1,550
Barnstead PM-70CB conductivity meter with cell	0	0	525
Spinco type 75-Ti rotor	0	0	1,700
New Brunswick G76 Gyratory shaker and 3 platforms	0	0	1,000
Kelvinator -96° UC-540 chest freezer	0	0	2,250
+ 6% California sales tax	1,616	251	470
Category total	28,546	4,441	8,295
Consumable supplies			
Replacement drive for Spinco L3-50	500	0	500
Tissue culture media and supplies	3,000	3,500	4,000
Chemicals and biochemicals	2,000	2,300	2,700
Radioactive materials	3,500	3,750	4,000
Animals and animal boarding in U. C. I. Vivarium	6,000	7,000	8,000
Glass- and plasticware	1,000	1,250	1,500
Radioactive counting supplies	1,000	1,250	1,500
Radioactive waste disposal	250	350	450
+ 6% California sales tax (not for animal boarding)	795	924	1,059
Category total	18,045	20,324	23,709
Miscellaneous			
Service contract for Spinco L3-50	300	350	400
Artwork, photography, page costs, reprints	1,000	1,250	1,500
Repairs and maintenance on laboratory equipment	1,250	1,500	1,750
Category total	2,550	3,100	3,650
Direct cost total	65,420	45,589	54,140
Three year direct cost total			$165,149

†Number of personnel working on this project: 10. W. M. Stanley, Jr., Witte, M. E., Brown, G. E., Kolb, A. J., Cashion, L. M., Cooper, K. D., Henderson, B., Neal, P., Dickinson, L. and Noble, S.

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Justification for personnel:

Wendell M. Stanley, Jr. (Principal Investigator): Salary for the 3 summer months is requested in order that the continuity of the project be assured. No summer vacations will be taken. Also working through the summer months will be a full time technician, 3 (and perhaps 4) graduate students, and undergraduate employees (see below).

Marcia Witte (Full time laboratory assistant, presntly at step III): Ms. Witte holds a B.S. in Biology from the School of Biological Sciences, Univ. of Cal., Irvine. She has been a technician in my laboratory for 3 years and is fully trained in the operations in the laboratory. She is paricularly essential for the care of fertile eggs and chicks, bleeding chicks by cardiac puncture, working up virus preparations, preparing ribosomes, ribosomal subunits, and cell extracts, preparing samples for radioactive counting, and in the preparation of reagents and solutions. Her continued employment is considered essential for the smooth progress of this project; another technician hired at a later date would take a considerable amount of my time in explaining the nature of the project and in training him/her for his/her duties. It also would be difficult to find a replacement technician with the biological education of Ms. Witte.

Laboratory Helpers: Three part time student laboratory helpers are requested to perform duties which do not require the specialized training of a laboratory technician. Such duties include gathering and transporting dirty glass- and plasticware, putting clean glass- and plasticware back where is belongs, maintaining the laboratory in a satisfactory state of cleanliness, cleaning the chicken brooders and egg incubator, and feeding and watering the chicks and chickens. Laboratory helpers also will assist other laboratory personnel whenever such help is consisent with the abilities of the student helpers.

Justification for permanent equipment:

The California Division of the American Cancer Society ($10,000, "Control of Protein Biosynthesis in Mammalian Cells", 7/1/68-6/30/69), the Cancer Research Coordinating Committee of the Univ. of California ($5,000, "Control of Protein Synthesis in Normal and Tumorous Cells", 7/1/68-6/30/69), and the American Cancer Society ($84,583, "Control of Protein Biosynthesis in Mammalian Cells", 3/1/69-6/30/71) originally provided the funds to equip my new laboratory when I moved to the Irvine campus of the Univ. of California. Although the original research project partially overlaps the project which I am presently proposing, there are sufficient differences to require new and additional equipment.

Beckman LS-330 scintillation spectrometer: Many of the experiments proposed in this application will be of the double radioactive label variety (see the subsection entitled "Characterization of products synthesized in vitro under the direction of wild-type and mutant AMV RNA"). The use of a scintillation spectrometer therefore is a necessity. There are 2 single channel scintillation counters in the department; they are in the laboratories of newly recruited staff members. These counters were purchased when the Irvine campus opened about 7 years ago and they are used

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for teaching purposes. In 1967, classes were taught once each year and the averge class enrollment was about 35; this year corresponding classes are taught 3 times a year and the enrollment per class has soared to between 150 and 200. The departmental counters are old, often out of service awaiting repair, heavily used by class students, and cannot be depended upon for research purposes. The Beckmodel LS-330 is a very practical machine, stripped of non-essential gadgets such as digital presentation (replaced with a much cheaper ratemeter) and automatic quench correction. The LS-330 has 3 channels and quench corrections can be made either by external standard or by channels ratio. Data output is by teletype (serviced by Beckman, and not by the very troublesome teletype field engineers) and punched paper tape which is compatible with the computor services available at Irvine for the analysis of double label experiments.

Leeds and Northrup 7415-E2 research pH meter: An expandable scale pH meter of high sensitivity is required for the accurate determination of pH of tissue culture media and of other solutions. This model is cheaper than digital display instruments but does not sacrifice reproducibility or accuracy. The E2 designation indicates that the instrument will be equipped with a miniature combination electrode for the pH measurement of small samples. The 7415 also is combatible with specific ion electrodes which may be purchased later, especially for the measurement of divalent cation concentrations which are so important in in vitro amino acid incorporation systems.

Amico DC2 hollow fiber dialyzer/concentrator: This instrument will be used to remove ammonium sulfate after precipitation of AMV, to remove sucrose and to concentrate AMV following equilibrium centrifugation of the virus in preformed sucrose density gradients, and to concentrate suspensions of the purified virus. Many of the proteins to be purified are very labile in dilute solution. Normal dialysis leads to denaturation and extensive losses of activity and, in addition, must be preceded or followed by concentration steps which in themselves often result in denaturation and losses of activity. The DC2 also will be used to dialyze and concentrate fractions obtained from ion exchange chromatography, gel filtration, isoelectric focusing, and preparative polyacrylamide gel electrophoretic purification of viral components and of protein synthesis initiation factors. The instrument will be used to concentrate and adjust the ionic composition and concentration of eukaryotic polysomal high salt washes, which are the starting fraction for eukaryotic protein synthesis initiation factors, prior to further purification steps. The great advantage of the DC2 is that the processes of dialysis and concentration are simultaneous, efficient, gentle, and very fast.

CRC Labwasher: Although the Media Kitchen at Irvine washes glass- and plasticware on a recharge basis, the rates are high (and getting higher every year), the losses due to breakage and theft are excessive, and, due to inefficiency, approximately ¼ of the laboratory glass- and plastic-ware is out of use while it is being processed by the Media Kichen. My laboratory operates throughout weekends and holidays, but the Media Kitchen does not. This results in an unacceptable pileup of dirty glass-

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and plasticware during such periods. I have conducted a cost analysis covering a 2 year period, and find that the use of a laboratory dish washer, which we can operate ourselves, actually would save money after a period of use of between 7 to 8 months. In addition, the glass- and plasticware would be cleaner (student help in the Media Kitchen is rather casual and we have been troubled by glass- and plasticware returned in an obviously dirty and unacceptable condition, and by residual detergents on glassware used for tissue culture). Washing our own glass- and plasticware would effectively increase the laboratory supply by a significant fraction, since there would be no pileups of dirty glass- and plasticware. We have already switched from the Media Kitchen to our own apparatus for the washing of pipetts; the results have been entirely satisfactory. Radioactively contaminated glass- and plasticware will continue to be processed by the Media Kitchen, since they have special equipment for the handling of such items.

New Brunswick T-41 roller bottle apparatus for tissue culture: At the present time all of our tissue culture work is being done in glass and plastic flasks. For the large scale growth and production of AMV and of AMV-producing cells, a larger capacity system is required. The roller bottle apparatus is the most efficient means to this end.

Two New Brunswick CO-20 CO_2 incubators: As stated above, our tissue culture work is now performed in flasks which are purged with air containing 5% CO_2, sealed, and incubated at 37° in an incubator with a normal atmosphere. For plaque assays in dishes with loose covers, which must be removed periodically for observation, and then returned for further incubation, and for the establishment of primary and secondary cultures of chick embryo and bone marrow cells, the present arrangement is clearly unsatisfactory for work at 2 temperatures: 2 separate incubators are required.

Spinco SW-40-Ti rotor: This rotor centrifuges tubes 3 3/4 inches in length and offers the highest resolution available for zone velocity analyses through preformed sucrose density gradients. It is required for the analysis of the RNA prepared from AMV, for the separation of the high molecular weight 70S AMV RNA from the endogenous lower molecular weight RNA species, for the analysis of high molecular weight 70S AMV RNA purified by adsorption to, and elution from, microcrystalline cellulose, for the analysis of 70S AMV RNA before and after denaturation, and for the analyses of radioactive AMV RNA bound to ribosomes and ribosomal subunits and of radioactive AMV RNA which has entered polysomes. The rotor is able to centrifuge 6 tubes, thus allowing the analysis of several samples at once. The high centrifugal forces developed shorten the duration of the experiment and analyses of high molecular weight RNA, which are particularly vulnerable to traces of RNAase, require the use of such a rotor.

Second year -- Sorvall RC2-B centrifuge with SS-34, GSA, and GS-3 rotors: The 2 departmental RC2-Bs have suffered the same fate described for the departmental scintillation counters discussed above. The SS-34 rotor holds 50 ml tubes, the GSA rotor 250 ml bottles, and the GS-3 rotor 500 ml bottles. This centrifuge, noted for its reliability, and the 3 rotors are requested for the harvest of large quantities of virus, for the separation of phases during the phenol extraction of AMV RNA, for the harvest of myeloblasts and other cells from chickens and from tissue culture, and for the clarification of cell extracts. This piece

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of equipment is postponed until the second year because quantities of AMV, myeloblasts and other cells large enough to require its routine use are not expected to be ready until after the first year, although it would be very useful immediately during the phenol extraction of the RNA of AMV.

Third year -- VW&R vacuum pump: The vacuum pump in the laboratory lyophilizer was purchased used and it has been under constant use for 4 years. The last time that it was rebuilt and serviced, I was told that its life expectancy was no more than another 2 or 3 years. The new pump will replace the present pump in the lyophilizer.

Amicon MMC concentrator: Although this 8-place concentrator could be used immediately, its purchase has been set off until the third year. This piece of apparatus is designed to remove sucrose and concentrate samples recovered from sucrose gradients. It would be especially useful for the preparation of AMV RNA samples of varying sedimentation coefficient. It is fast in operation and this property is important during the processing of high molecular weight RNA. The samples that it accepts are small (10 ml); its use would be highly preferable to the usual practice of dialysis followed by ethanol precipitation.

Barnstead PM-70CB conductivity meter plus cell: This meter will be used to monitor the salt concentration during gradient elution of viral components from columns of ion exchangers, during gradient elution of viral RNA from microcrystalline cellulose, and for similar applications. It will also be used to check the concentrations of various reagent stock solutions. This instrument also would be useful immediately.

Spinco type 75 rotor: This angle rotor develops sufficient centrifugal force to sediment the 70S RNA from AMV in about 1 hour. It is therefore very useful for the recovery of such RNA from various solutions. The department has 1 65 rotor, but it is nearing the time when it must be derated. The purchase of this rotor has therefore been postponed until the third year.

New Brunswick G76 Gyratory water bath shaker with 3 platforms: This piece of apparatus, noted for its reliability, is requested for the incubation of tissue culture flasks during short term labeling experiments of AMV producing-myeloblasts and for the incubation of yeast cultures from which L-[^{35}S]methionine of very high specific activity is produced. This instrument could also be useful immediately.

Kelvinator -96° chest freezer UC-540: We presently have a Kelvinator upright -70° freezer. Although it is defrosted, cleaned, and materials no longer useful discarded, it has been in use for 4 years and its capacity has been reached. The chest freezer is requested for long term storage so that the upright freezer can be used for shorter term storage. This freezer could be put into service immediately.

Justification for consumable supplies:

The expenditures for consumable supplies are mostly self-explanatory. However 2 items might be worthy of some explanation.

Replacement drive for the Spinco L3-50: These drives are requested every 2 years; that is the average life-time of the L3-50 drive. Without such replacement drives, the L3-50 (purchased with A. C. S. funds from an earlier grant) would become useless.

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Animals and animal boarding in the U. C. I. Vivarium: The actual purchase price of animals is but a small % of this item. Vivarium costs at U. C. I. (animals must be kept within the Vivarium; there is no alternative) are extremely high; over the past few years they have increased dramatically, and they undoubtedly will go higher yet. Arguments with the Vivarium personnel and with the administration have come to naught.

Economies made in the area of consumable supplies:

Care of eggs, chicks, and chickens: Laboratory personnel care entirely for the eggs and these animals; we purchase the feed and the bedding, clean the incubator and brooder (which we originally purchased), and clean the room in which they are kept. The Vivarium has the grace to only charge us half price for the maintenance of the animals under these conditions. At least we save half.

tRNA: We isolate our own tRNA from chicken liver, chicken reticulocytes, rabbit liver, rabbit reticulocytes, rat liver, and chick embryos and myeloblasts. It is true that it is cheaper than commercial materials, but the main reason is that our product is much purier and more active.

L-[^{35}S]Methionine: We produce this material in the laboratory because it is much less expensive than the commercial products (1/50), but the main reason is the same one as given above; the product is isotopically pure -- commercial samples are grossly contaminated with methionine sulfoxide and several unknown sulfur-containing compounds.

Consultants in the area of the project proposal:

Dr. Gale Granger will offer advice and counsel in the area of immunology; this is his field of expertise. He is in the same department as I.

Dr. Charles Gordon will offer his services, advice and counsel in the area of electron microscopy; this is his field of expertise. He is in the same department as I.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
In Vitro Translation of Oncogenic C-Type Viral RNA	Cancer Research Coordinating Committee of the University of Calif. UCI-1719	12,000	7/1/73-6/30/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Temperature Sensitive and Conditional Lethal Mutants of Avian Myeloblastosis Virus	Calif. Div. of the ACS UCI-1723 Grant deferred -- Agency has no available funds.	30,364	9/1/73-8/31/74
Isolation and Characterization of Temperature Sensitive and Conditional Lethal Mutants of Avian Myeloblastosis Virus and the Identification of Virus-Specific Products Synthesized in Vitro under the Direction of Wild-Type and Mutant Viral RNA	Am. Cancer Society UCI-1730 Grant deferred -- Insufficient funds at present -- will be considered later for possible funding as of 7/1/74	167,256	1/1/74-12/31/76
Studies on Chicken Leukemia Viruses	Damon Runyon Mem. Fund This grant seeks support for graduate students and for postdoctoral students.	98,668	9/1/73-8/31/75
Biochemical and Genetic Studies on AMV	Jane Coffin Childs Mem. Fund This grant asks support for grad. & postdoctoral students.	63,982	9/1/73-8/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator Wendell M Stanley Jr

Typed Name Wendell M. Stanley, Jr.

Signature Wendell M Stanley Jr Date July 31, 197

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Mailing address for checks

Responsible officer of institution

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Title

Signature Date

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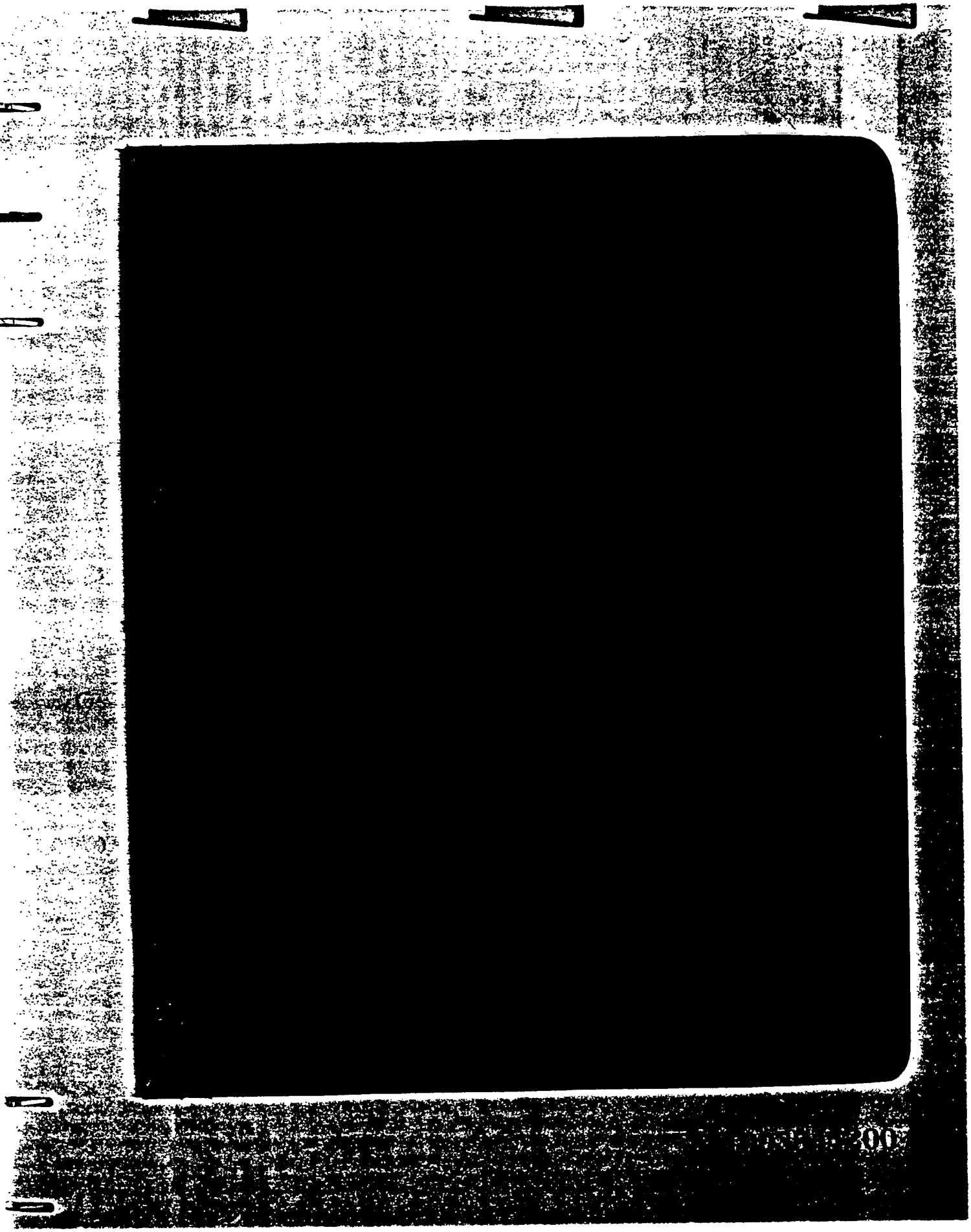

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